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TITLE: The Effect of Cancer Chemopreventive Agents on DNA Adduct
Formation by the Dietary Prostate Carcinogen PhIP

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13. ABSTRACT (Maximum 200 Words)

This goal of this proposal was to investigate chemopreventive strategies to reduce the genotoxic effects of the prostate carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). PhIP is considered to pose a significant prostate cancer risk to humans because it is found in cooked meat and epidemiology studies have linked meat consumption to prostate cancer. Importantly, PhIP causes prostate cancer in rats following high-dose exposures. Our purpose was to use the rat model to determine the risk posed by PhIP at levels found in the diet and to identify candidate chemopreventive agents for PhIP. Over the period of this grant, we have established that PhIP forms DNA adducts in the prostate and DNA adduct levels are dose-dependent. We have identified PEITC and chlorophyllin as potential chemopreventive agents for PhIP and have established potential mechanisms of action. Wine, quercetin, genistein and lycopene have also been investigated, although they did not reduce adduct formation. Follow-on funding has been obtained from NIH, in which we will determine if PEITC and chlorophyllin reduce PhIP-induced prostate tumor formation. We also hope to translate this research in to a treatment that can be used in people (a recently submitted NIH Spore grant with the UC Davis Medical Center, Sacramento, CA).

14. SUBJECT TERMS
Prevention, Cancer, Biomarker, Prostate Accelerator Mass Spectrometry

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Introduction:

The goal of this proposal was to investigate chemopreventive strategies to reduce the genotoxic effects of the prostate carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). PhIP is considered to pose a significant prostate cancer risk to humans because it is found in relatively high concentrations in cooked meat (Zhang *et al.*, 1988; Felton and Knize, 1990) and some epidemiological studies have shown a correlation between meat consumption and prostate cancer incidence (Mills *et al.*, 1989; Talamini *et al.*, 1992; De Stefani *et al.*, 1995; Ewings and Bowie, 1996). Importantly, PhIP causes prostate cancer in rats following high-dose exposures (Shirai *et al.*, 1997). However, studies to establish carcinogenicity and determine the efficacy of chemopreventive agents generally employ chemical doses orders of magnitude higher than the average human daily intake, hence are of questionable relevance. Therefore, our purpose was to use the rat model and the highly sensitive technique of accelerator mass spectrometry (AMS) (Turteltaub *et al.*, 1990a) to determine the risk posed by PhIP at levels found in the diet. We used DNA adduct formation by PhIP as a measure of risk, as it is a form of DNA damage which is considered an early event in the development of cancer. Subsequently, we aimed to identify candidate chemopreventive agents and the effective doses that could be used to reduce DNA adduct formation, and consequently prostate cancer risk, as a result of dietary exposure to PhIP.

Body:

The progress made towards the specific aims of the research project completed in the period April 1, 2000 to March 31, 2003 is described as follows:

Specific aim #1: Determine the bioavailability of PhIP and the level of adduct formation in rat prostate following dietary levels of exposure.
The goal of this aim was to establish if PhIP forms adducts with DNA, the effect of dose and the kinetics of formation and clearance of DNA adducts. Furthermore, in order to investigate the utility of blood protein adducts as a surrogate biomarker of DNA adduct formation in the prostate, adduct formation with albumin was analyzed.

In an experiment to establish how much PhIP reaches the prostate and the level of adduct formation, we established that the dose-responses were linear with no apparent threshold at low doses. This indicates that risk from exposure might be extrapolated from the high doses of PhIP that cause tumors in rats, to dietary levels of exposure. The amount of PhIP measured in the tissues is illustrated in figure 1 and the DNA adduct levels in figure 2.
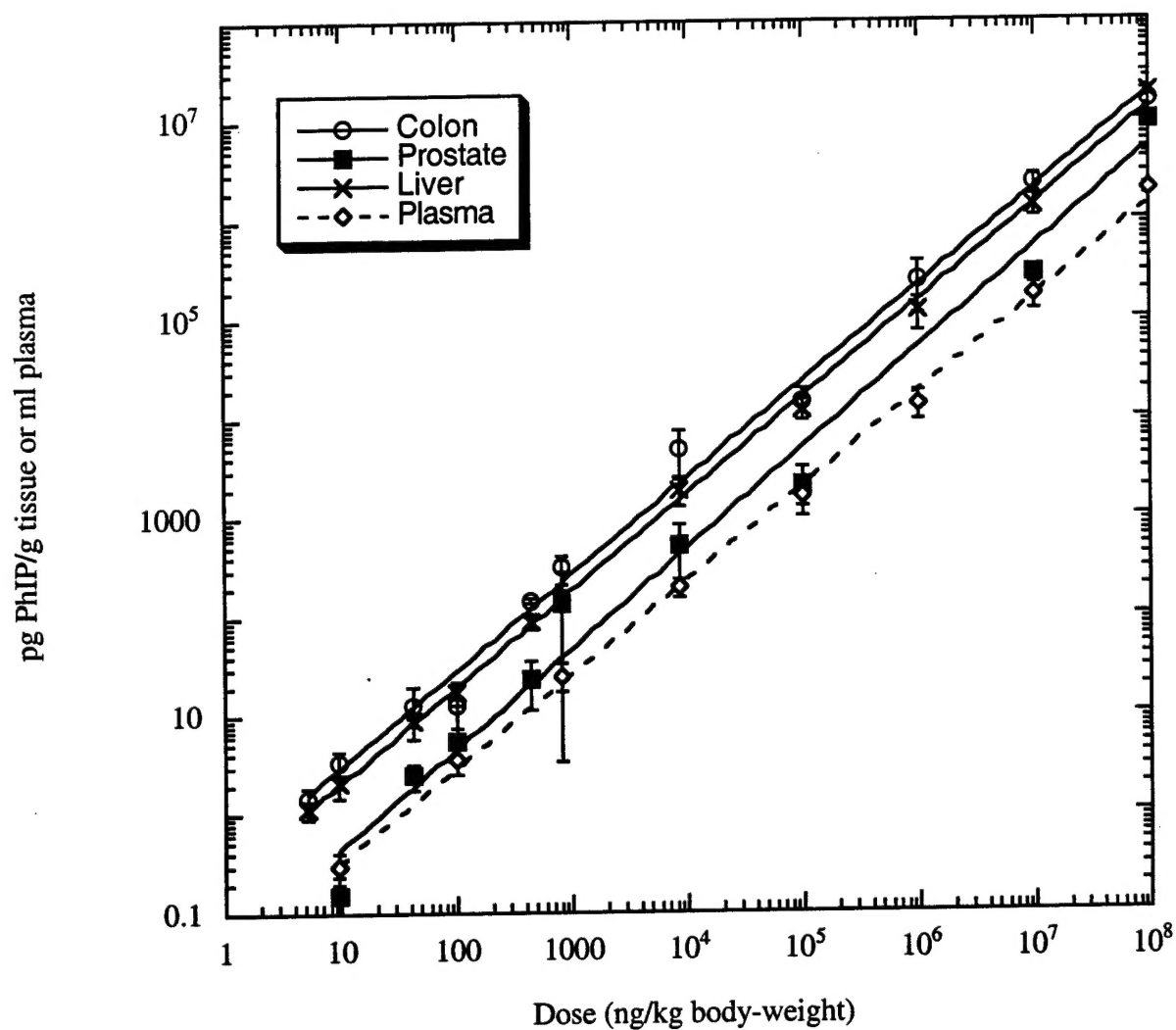
Figure 1. The amount of PhIP measured in the colon, prostate, liver and plasma following exposure in the dose range 10 ng-100mg/kg body-weight [^{14}C]PhIP.
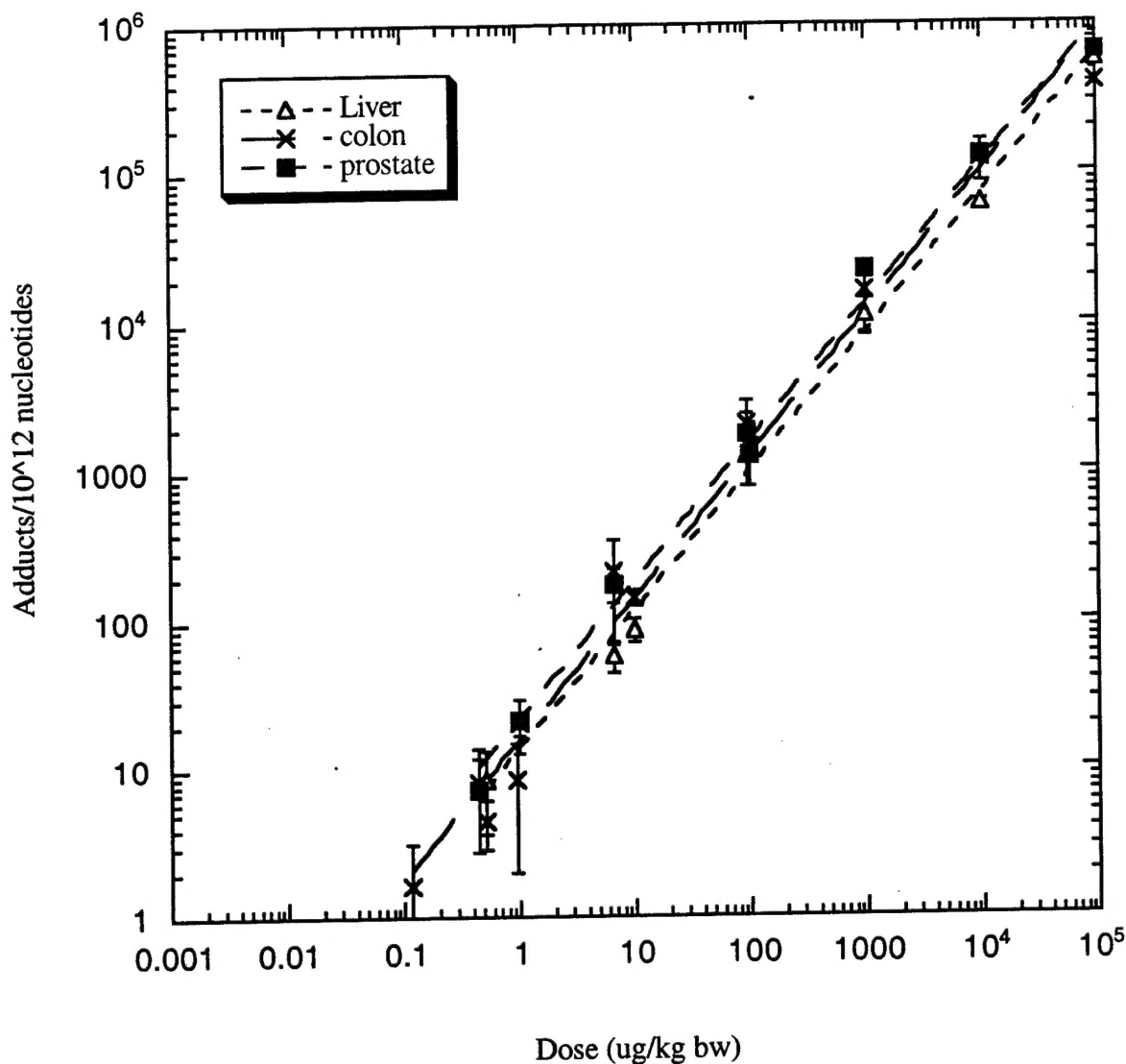
Figure 2. PhIP-DNA adduct levels in the prostate, colon and liver of rats following exposure in the dose range 10 ng-100mg/kg body-weight [^{14}C]PhIP.

Furthermore, the dose-response for albumin adducts in the blood was also linear, which indicates that albumin adducts may be a good biomarker of PhIP exposure and bioactivation for use in molecular epidemiology and chemoprevention studies (figure 3). In a separate experiment, we showed that adducts were also still present in the prostate and blood up to 72 hours after exposure and that the prostate consistently had higher adduct levels than the liver or colon.
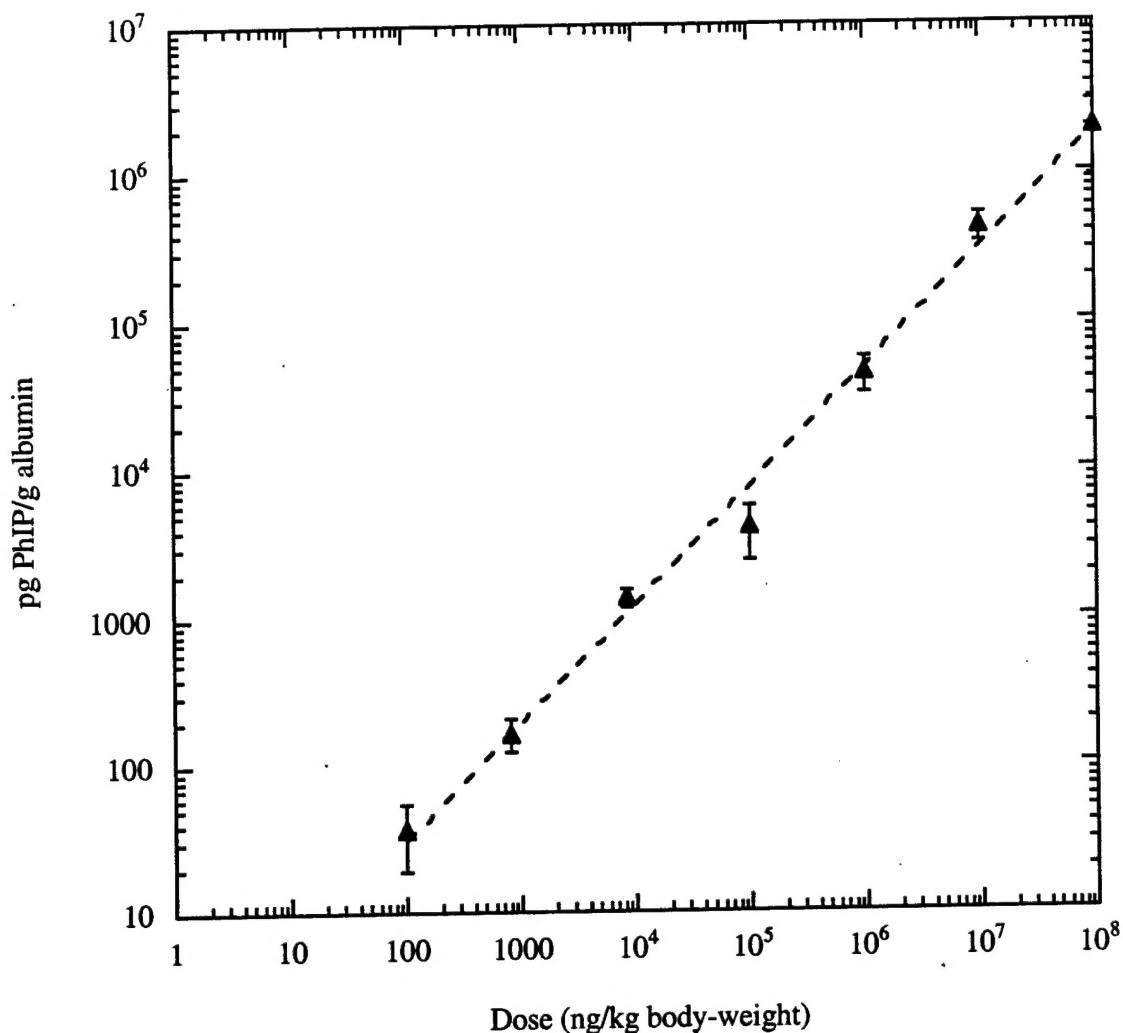
Figure 3. The amount of PhIP bound to albumin in the blood following exposure in the dose range 100 ng-100mg/kg body-weight [^{14}C]PhIP.

The data from this aim will be included in a manuscript, titled ('Metabolism And DNA Adduct Formation Of 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine (PhIP) At Low Dose In The Male F344 Rat'). The methodology for isolation of the DNA and analysis by AMS was included as a book chapter ('DNA isolation and sample preparation for quantitation of adduct levels by Accelerator Mass Spectrometry'. Dingley, K.H., Ubick, E.A., Vogel, J.S. and Haack, K.W. In: Keohavong, P. and Grant, S.G. (eds.) Protocols in Molecular Toxicology, Humana, Totowa, NJ. Methods in Molecular Biology (in press) (see appendix for manuscript).

Specific aim #2: Determine the metabolism of PhIP in rat prostate at dietary levels of exposure. In year 1 we established that circulating PhIP metabolite, N-OH PhIP, may be a factor in the targeting of PhIP to the prostate.

Plasma metabolites were analyzed by HPLC at defined time points between 0.5 and 24 hours following PhIP exposure. Representative results from analysis of HPLC fractions by AMS at 12 hr are shown in figure 4. Eight radiocarbon containing peaks were detected (at least 10-times above background) at each of the time points measured. The identification of the plasma metabolites was made by matching the retention times of the radiocarbon peaks to those of authentic metabolite standards purified from rat urine following a 50 mg/kg dose of PhIP (Table 1).
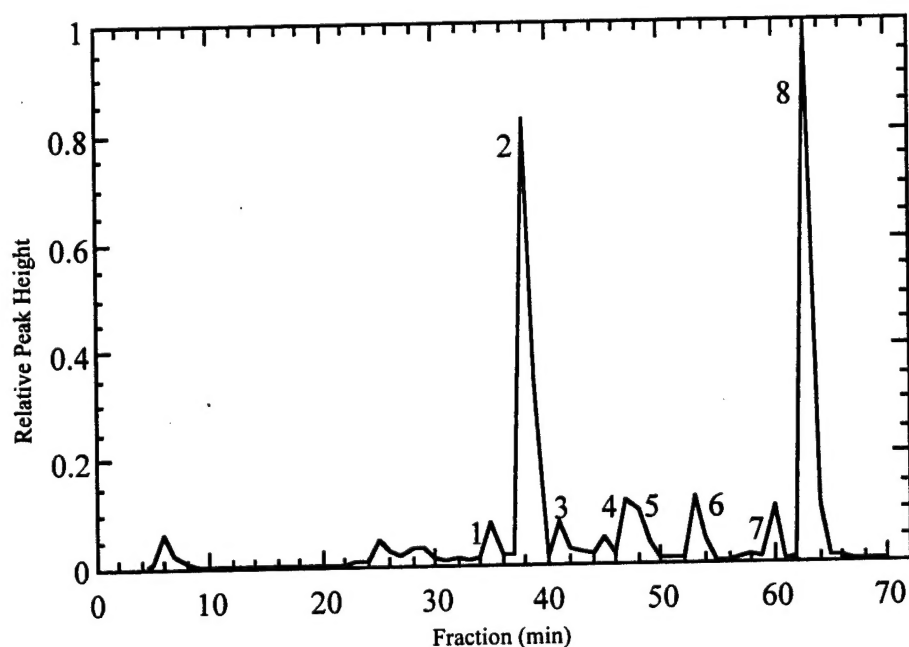
Figure 4. PhIP plasma metabolite profile from the 12 hour time point.

Table1. Metabolite Identification

Peak No.	Ret. Time (min)	$(M+H)^+$	Metabolite
1	31.5	401	4'-O-glucuronide
2	34.8	321	4'-PhIP-SO$_4$
3	37	---	unidentified
4	42.9	417	N-OH,N$_2$-glucuronide
5	44.4	241	4'-OH-PhIP
6	49.4	417	N-OH,N$_3$-glucuronide
7	57.6	N/A	N-OH-PhIP
8	60.1	225	PhIP

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PhIP was the major component present in plasma at all time points followed by 4'-PhIP sulfate. Interestingly, a radiocarbon peak detected at 57 minutes corresponded to the retention time of N-OH-PhIP. This putative N-OH-PhIP metabolite was only found in rat plasma. To eliminate the possibility that this peak was due to other PhIP metabolites with similar retention characteristics, an isocratic HPLC method was developed which resolves N-OH-PhIP, NO_2-PhIP, and PhIP. Using the isocratic method, the putative N-OH-PHIP peak seen in plasma eluted at the same time as the N-OH-PhIP authentic standard. While this is preliminary, it suggests that N-hydroxy-PhIP does circulate and does not need to be produced deNovo in the extra hepatic tissues. To our knowledge this is the first evidence that N-hydroxy-PhIP circulates following administration of PhIP to a whole animal. N-hydroxy-PhIP is the result of PhIP oxidation by cytochrome P4501A2, an enzyme found in the liver. N-hydroxy-PhIP is a potentially genotoxic metabolite which can be esterified via Phase II enzymes to form either N-acetoxy-PhIP N-sulfoxy-PhIP, metabolites thought to be responsible for the formation of PhIP-DNA adducts.

In year 2, we investigated if the prostate has the capacity to metabolize [^{14}C]PhIP to genotoxic metabolites, which would help determine which pathways of PhIP metabolism could be targeted in human prostate cancer chemoprevention. Microsomes prepared from rat prostate did not generate oxidation products of PhIP. In contrast, liver microsomes generated multiple oxidation products from PhIP, including NOH-PhIP, an intermediate that binds to DNA. We concluded that NOH-PhIP is not generated by cytochrome P450 at detectable levels in the prostate but is supplied to the prostate by the circulation.

Specific aim #3: Determine the effect of chemopreventives on PhIP bioavailability, metabolism and adduct formation in the prostate.

The goal of this aim was to identify several candidate chemopreventive agents and the effective doses that could be used to reduce human prostate cancer risk as a result of dietary exposure to PhIP.

A pilot study was conducted in year 1 investigating the effect of wine, quercetin, PEITC (phenylethylisothiocyanate) and sulforaphane on adduct formation by PhIP. We showed that the isothiocyanate PEITC reduced adduct formation in the prostate. Although preliminary, these results suggested that PEITC had a protective effect. This data has been included in a manuscript that will be presented at the 5th International Congress on Wine and Health in Marsala Italy, May 8-11, 2003 and will be published following the meeting ('Animal Models And Analytical Approaches For Understanding The Relationships Between Wine And Cancer'. See appendix).

The study was repeated in year 2 using larger groups of animals, a lower dose of [^{14}C]PhIP (8 μg/Kg body weight (bw)) and 2 doses of PEITC to look at the effect of a lower dose (816 mg/Kg and 82 mg/Kg in diet). We also examined the effect of genistein (found in soy; 500 mg/Kg diet), chlorophyllin (a stable, sodium salt of chlorophyll; 1% w/w in diet) and lycopene (as the dietary supplement Lycovit; 0.1% w/w lycopene in diet). DNA adduct levels were measured in the liver, colon and prostate. The data has been included in a manuscript that has been submitted to Nutrition and Cancer (Effect of Dietary Constituents with Chemopreventive Potential on Adduct Formation of a Low

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Dose of the Heterocyclic Amines PhIP and IQ and Phase II Hepatic Enzymes See appendix). Also included in the manuscript, is data collected in year 3 of the grant in which we investigated the effect of PEITC, genistein, chlorophyllin and lycopene on phase II detoxification enzyme activities.

To establish if lower doses of PEITC also reduced adduct levels following PhIP exposure, we had reduced the amount of PEITC in the diets of the rats by 10-fold to 82 mg/kg body-weight. We found no statistically significant reduction in adduct levels, however, there was a significant increase in the activity of the phase II detoxification enzyme glucuronyltransferase. The lack of adduct reduction implies that an exposure dose of PEITC that is more representative of human dietary intake may not be effective in reducing prostate cancer risk following exposure to PhIP.

In year 2, the mechanism by which PEITC reduces the carcinogenic potential of PhIP was also investigated. To determine if PEITC interferes with PhIP bioactivation, microsomes from rat liver were prepared to measure the metabolism of PhIP in the presence of PEITC. PhIP metabolism by liver microsomes was inhibited by PEITC. With particular significance, PEITC abolished the production of NOH-PhIP, while less reactive metabolites were still produced. We conclude that PEITC inhibits cytochrome P450 dependent metabolism of PhIP and that inhibition of the metabolic activation of PhIP to reactive intermediate such as NOH-PhIP may lead to the reduction of DNA adducts.

In order to investigate whether this chemoprevention mechanism may also apply to humans, the effect of PEITC on PhIP bioactivation was tested in vitro using recombinantly expressed human and rat cytochrome P450 1A1/1A2. The bioactivation of PhIP by expressed cytochrome P450 1A1 /1A2 produced two predominant metabolites, 4'OH-PhIP and NOH-PhIP. The ratio of 4'OH/NOH metabolites produced differed between individual CYP450 isoforms and between rat and human. When PEITC was added to incubations containing expressed proteins, the bioactivation of PhIP was inhibited. The inhibition appeared to be dose responsive as greater concentrations of PEITC inhibited PhIP bioactivation to a greater degree. We concluded from these studies that metabolism of PhIP differs greatly between human and rat P450 isoforms and PEITC inhibits bioactivation of PhIP.

To investigate the effect of PEITC on PhIP detoxification, rats were fed PEITC for nine days. Twenty-four hours later, microsomes from rat liver were prepared to determine if glucuronidation was induced. When rats were exposed to dietary concentration of PEITC over nine days, glucuronidation of NOH-PhIP was induced. Compared to control, rats fed PEITC appeared to induce detoxification of NOH-PhIP by glucuronidation approximately two-fold. Therefore, PEITC appears to induce the major route of detoxification for PhIP.

Key Research Accomplishments:

During this grant period, we have shown that:
1. PhIP, a prostate carcinogen, is bioavailable to the prostate at dietary levels of exposure and the levels in the prostate are dose-dependent.
2. PhIP damages DNA in the prostate through formation of DNA adducts. Adduct levels are dose-dependent.
3. PhIP forms dose-dependent levels of albumin adducts in the blood. Albumin adducts may be a good biomarker of exposure to PhIP and of DNA adduct formation in tissues.
4. In rats, PhIP is bioactivated to N-OH PhIP, which circulates in the blood and may lead to DNA adduct formation in the prostate.
5. Rat prostate does not have the capacity to activate PhIP to N-OH PhIP by cytochrome P450. This metabolite most likely reaches the prostate via the blood.
6. PEITC and chlorophyllin reduce adduct formation in the prostate, as well as other organs.
7. The inhibition of adduct formation by PEITC is dependent on dose.
8. Wine, quercetin, sulforaphane, genistein and lycopene did not significantly reduce adduct formation by PhIP in the prostate.
9. Albumin adduct levels in blood reflect DNA adduct levels in the prostate and is good indicator of chemopreventive effect in the prostate.
10. PEITC and chlorophyllin may reduce adduct formation in the prostate via alterations in PhIP metabolism.

Reportable Outcomes:

1. **Abstracts/poster presentations**
    1. Posters entitled 'The effect of dietary supplements with chemopreventive potential on metabolism and DNA-adduct formation of the heterocyclic amine PhIP in rats' were presented at the UC Davis Cancer Center Research Symposium, October 6-7, 2000 and the Federation of American Societies for Experimental Biology (FASEB) meeting, March 31-April 4, 2001. The abstract was published in FASEB J. (2001), 15(4), A618 Part 1.
    2. Abstract entitled 'Phenylethylisothiocyanate (PEITC) both inhibits cytochrome P450 1A1/1A2 bioactivation and induces glucuronide detoxification of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)'. Presented by West, J.A., Turteltaub K.W., and Dingley K. at the Society of Toxicology Annual Meeting, Nashville, TN., March 17-21 2002.
    3. Abstract entitled 'Phenylethylisothiocynate (PEITC) reduces 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) DNA adduct formation by inhibition of Cytochrome P450 dependent metabolism'. Presented by West, J.A., Turteltaub K.W., and Dingley K. at the Society of Toxicology Annual Meeting, Nashville, TN., March 17-21 2002.

4. Abstract entitled 'Characterization of the protein adducts formed by the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)' was presented by Cindy Lou Chepanoske, Karen Brown, Kenneth Turteltaub and Karen Dingley at the Mass Spectrometry in Biology and Medicine conference, San Francisco, CA, 2001.

2. **Oral presentations**
   1. This work was presented as an oral presentation as part of DOE science days at Lawrence Livermore National Laboratory, March 22-23, 2001.
   2. Presentation Entitled 'Macromolecular Adduct Formation In Humans And Rodents Following Exposure To Dietary Doses Of PhIP' was presented by Ken Turteltaub at the 8th International Conference on Carcinogenic/Mutagenic N-Substituted Aryl Compounds, November 12-14, 2001.
   3. Presentation entitled 'Effect of dietary supplements with chemopreventive potential on adduct formation following a low dose of PhIP' was presented by K. Dingley at the 8th International Conference on Carcinogenic/Mutagenic N-Substituted Aryl Compounds, November 12-14, 2001. K. Dingley received a **Research Award for Outstanding Contribution of Original Research**.
   4. Presentation entitled 'Adduct formation by heterocyclic amine carcinogens following dietary-relevant doses' was presented by Karen Dingley at the 223rd National Meeting of the American Chemical Society, Orlando, FL, April 7-11, 2002.
   5. A talk entitled 'Animal Models And Analytical Approaches For Understanding The Relationships Between Wine And Cancer' will be presented by Sue Ebeler at the 5th International Congress on Wine and Health in Marsala Italy, May 8-11, 2003.

3. **Publications**
   1. Dingley,K.H., Ubick, EA., Chiarappa-Zucca, ML., Nowell, S., Abel, S., Ebeler, SE., Mitchell, AE, Burns, SA., Steinberg, FM., and Clifford, AJ. (2001)The effect of dietary supplements with chemopreventive potential on metabolism and DNA-adduct formation of the heterocyclic amine PhIP in rats'. *FASEB J.*, **15(4)**, A618 Part 1.

   2. West, J.A. Dingley, K.H., and Turteltaub, K.W. (2002) Phenethylisothiocyanate (PEITC) reduces 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) DNA adduct formation by inhibition of cytochrome P450 dependent metabolism. *Toxicological Sciences*, 66 1-S, 617.

   3. Dingley, K., Sanchez, E., Chiarappa-zucca, M., and Turteltaub, K. (2002) Accelerator Mass Spectrometry: A tool for tracing environmentally-relevant doses of ^{3}H and ^{14}C-labeled carcinogens. *Toxicological Sciences*, 66 1-S, 883.

   4. West, J., Turteltaub, K., and Dingley, K. (2002) Phenethylisothiocyanate (PEITC) both inhibits cytochrome P450 1A1/1A2 bioactivation and induces glucuronide

detoxification of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Toxicological Sciences*, 66 1-S, LB23.

5. Dingley, K.H., Ubick, E.A., Vogel, J.S. and Haack. DNA isolation and sample preparation for quantitation of adduct levels by Accelerator Mass Spectrometry. K.W. In: Keohavong, P. and Grant, S.G. (eds.) Protocols in Molecular Toxicology, Humana, Totowa, NJ. *Methods in Molecular Biology* (in press).

6. Dingley,K.H., Ubick, EA., Chiarappa-Zucca, ML., Nowell, S., Abel, S., Ebeler, SE., Mitchell, AE, Burns, SA., Steinberg, FM., and Clifford, AJ. Effect of Dietary Constituents with Chemopreventive Potential on Adduct Formation of a low dose of the Heterocyclic Amines PhIP and IQ and Phase II Hepatic Enzymes (submitted to Nutrition and Cancer; see appendix).

7. Ebeler, S.E., Dingley, K.H., Ubick, E., Abel, S., Mitchell, A.E., Burns, S.A., Steinberg, F.M. and Clifford, A.J. Animal Models and Analytical Approaches for Understanding the relationships between Wine and Cancer' (in preparation; see appendix for draft).

4. Employment/Research Opportunities
Over the period of this grant, we were able to hire a post-doc (Jason West) from the UC Davis Toxicology Program and a Summer student, Kristin Stoker from UC Berkeley, to work on this project. This was the first opportunity for Jason and Kristin to work in prostate cancer research.

Conclusions:

During this grant, we have made significant progress on our specific aims. We have determined that PhIP, a compound formed in meat during cooking, is bioavailable to prostate tissue following dietary-relevant doses. Importantly, exposure also results in DNA adduct formation in the prostate. DNA adducts are an early event in the development of cancer, hence these results imply that PhIP consumption is a cancer risk at dietary levels of exposure in the rat. Analysis of blood for protein adduct formation also indicated that albumin adducts may be a useful biomarker of PhIP exposure and adduct formation in the tissues for use in molecular epidemiology studies. Follow-on funding for characterization of the albumin adducts and development of an assay for measuring them in humans has been obtained from the DOD prostate cancer program (DAMD17-03-1-0076).

To better understand why PhIP may target the prostate, we have investigated the pathways of PhIP metabolism in the rat. Our results suggest that PhIP is activated to NOH-PhIP (probably primarily in the liver), which then circulates in the blood. This

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metabolite is important, as it is considered to be responsible for the formation of PhIP-DNA adducts. We found that prostate tissue was not able to activate PhIP to NOH-PhIP.

We have investigated several potential chemopreventive compounds for their ability to reduce adduct formation by PhIP in rats. PEITC, an isothiocyanate found in cruciferous vegetables, and chlorophyllin, a stable form of chlorophyll, both reduced adduct formation in prostate. Therefore they may be useful in the prevention of PhIP-induced prostate cancer in rats. Follow-on funding has been obtained via an NIH RO3 award (A preclinical model of prostate cancer) to investigate this hypothesis. Also, in order to translate this work in to treatments for prostate cancer in humans, we have submitted a project entitled 'Evidence Based Treatment for Prostate Cancer' as part of an NIH-SPORE project with UC Davis Cancer Center, Sacramento, CA.

"So What?"
As a result of the work completed over the past 3 years, we have made the following contributions to conquering prostate cancer:

1. Obtained further evidence linking the dietary prostate carcinogen PhIP to the development of human prostate cancer. This complements epidemiological studies that link meat consumption to prostate cancer risk.
2. Validated albumin adduct formation in the blood as a biomarker of dietary PhIP exposure and prostate cancer risk that could be used to identify individuals to be targeted for prevention and for monitoring the effect of chemoprevention strategies.
3. Identified PEITC and chlorophyllin as chemopreventive agents that could be used to reduce human prostate cancer risk as a result of dietary exposure to PhIP.
4. Identified mechanisms that may be involved in chemoprevention by PEITC and chlorophyllin. These mechanisms are likely to be relevant to humans, although dietary-relevant doses of PEITC may not be effective in chemoprevention *in vivo*.

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Appendices:

Attached are copies of manuscripts entitled:
1. 'DNA isolation and sample preparation for quantitation of adduct levels by Accelerator Mass Spectrometry' (in press).

2. 'Effect of Dietary Constituents with Chemopreventive Potential on Adduct Formation of a low dose of the Heterocyclic Amines PhIP and IQ and Phase II Hepatic Enzymes' (submitted to Nutrition and Cancer).
3. 'Animal Models And Analytical Approaches For Understanding The Relationships Between Wine And Cancer' (in preparation).

Below is the list of the primary personnel receiving pay from the research effort:

Name	Degree
Kenneth W. Turteltaub	Ph.D.
Karen H. Dingley	Ph.D.
Jay A. West	Ph.D.
Esther A. Ubick	B.S.
Marina Chiarappa-Zucca	M.S.
David O. Nelson	Ph.D.

DNA isolation and sample preparation for quantitation of adduct levels by Accelerator Mass Spectrometry

Karen H. Dingley, Esther A. Ubick, John S. Vogel and Kurt W. Haack

Abstract

A protocol is described for the isolation of DNA and subsequent preparation of samples for the measurement of adduct levels by Accelerator Mass Spectrometry (AMS). AMS is a very sensitive technique used for the quantitation of adducts following exposure to carbon-14 or tritium labeled chemicals, with detection limits in the adducts 10^{11}-10^{12} nucleotides range. However, special precautions must be taken to avoid cross-contamination of isotope between samples and to produce a sample that is compatible with AMS. The DNA isolation method is based upon digestion of tissue with proteinase K, followed by extraction of DNA using Qiagen DNA isolation columns. DNA is then precipitated with isopropanol, washed repeatedly with 70% ethanol to remove salt and then dissolved in water. This method has been used to reliably generate good yields of uncontaminated, pure DNA from animal and human tissues for analysis of adduct levels. For quantitation of adduct levels from ^{14}C-labeled compounds, DNA samples are then converted to graphite and the ^{14}C content measured by AMS.

Key Words

Accelerator Mass Spectrometry, DNA, Adduct, Carbon-14, Tritium

1. Introduction

Most known chemical carcinogens form reactive intermediates that are capable of reacting with DNA, forming covalent adducts. This damage may lead to mutations and ultimately cancer (reviewed in 1). Consequently, for risk assessment it is important to establish if drugs and toxicants form DNA adducts, particularly following doses that are encountered in every day life. Traditional methods used to monitor DNA adducts include ^{32}P-postlabeling, fluorescence techniques, GC/MS and immunoassays, with detection limits typically in the range of 1 adduct/10^7-10^9 nucleotides (reviewed in 2). Accelerator Mass Spectrometry (AMS) allows one to establish if chemicals form DNA adducts at even lower levels (adducts/10^{10}-10^{12} nucleotides range) through the use of carbon-14 (^{14}C) or tritium (^{3}H) labeled compounds (reviewed in 3). Thus DNA adduct levels can be established following low chemical doses or using compounds that that have a low covalent binding index (eg. 4-7). Due to the fact that the amounts of chemical and radioactivity required are low, such studies can be conducted safely in humans (4, 8-9).

AMS is a nuclear physics technique that can measure isotopes with a low natural abundance and long half life (eg. ^{14}C and ^{3}H) with high sensitivity and precision (reviewed in 10). It was originally developed for use in the earth sciences, but has now found widespread use in biology, with applications in areas such as cancer, nutrition and pharmaceutical research (reviewed in 1, 5, 11). However, as AMS is so sensitive, cross contamination of samples by isotope from equipment and laboratory supplies can be a major problem (12). Therefore, certain methods have been chosen to avoid contamination. For example, we have found that phenol/chloroform extraction, a process

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that is used frequently in DNA isolations, can be a major source of ^{14}C contamination. All gloves, tubes, forceps, and containers for buffers etc. must be disposable. Furthermore, before analysis by AMS, samples must be converted to graphite (for ^{14}C analysis) or titanium hydride (for ^3H analysis) (13-14). Therefore, the samples must be compatible with this process. For example, this necessitates the complete removal of sodium salts from the extracted DNA samples by repeated washing with 70% ethanol.

This chapter describes a protocol for extracting DNA from tissues for analysis of ^{14}C or ^3H content by AMS. The method is based upon the use of Qiagen columns (Qiagen Inc.). Procedures for contamination avoidance in samples are included throughout. After the section on DNA isolation, a description of the process for the conversion of the biological material to graphite for ^{14}C analysis is presented. AMS is then used to quantitate the amount of ^{14}C in the graphite samples. Due to the size and cost of an AMS instrument, this technique is not yet a routine tool in many laboratories. However, there are several facilities in the United States where samples can be sent for analysis. One such facility, The Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory, has a compact AMS system for the analysis of biological samples (15).

2. Materials

2.1   Tissue Homogenization and protein digestion

1. Plastic wrap (e.g. Saran wrap)

2. Aluminum foil

3. Parafilm

4. Polypropylene 50 ml tubes (e.g. Falcon brand from Becton Dickinson, Franklin Lakes, NJ)

5. Hammer and plastic bag to cover

6. Disposable scalpels

7. Disposable forceps (Cole-Parmer Instrument Company, Vernon Hills, IL)

8. Lysis buffer (4M urea, 1% Triton X-100, 10mM EDTA, 100mM NaCl, 10mM Tris-HCl pH 8.0, 10mM dithiothreitol)

9. Proteinase K (Sigma, St. Louis, MO), dissolved in double distilled water to 40 mg/ml

10. Shaking water bath or other mixer that will incubate at 37°C

2.2 RNA digestion

RNAse T1 (Sigma), diluted in double distilled water to 100 μg/ml

RNAse A (Sigma), dissolved in double distilled water to 10 mg/ml

2.3 Column purification

1. Qiagen Genomic tip-500 columns (Qiagen Inc., Valencia, CA) (see note 1)

2. 5M Sodium chloride

3. 1M 3-Morpholinopropanesulfonic acid (MOPS) pH7.0

4. Buffer B (750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0)

5. Buffer C (1M NaCl, 50mM MOPS, 15% ethanol, pH 7.0)

6. Buffer λ (1.25M NaCl, 50mM MOPS, 15% ethanol, pH 8.0)

7. 50 ml polypropylene tubes

8. Holders for 50 ml polypropylene tubes

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2.4   Precipitation of DNA

1. Isopropanol

2.5   Sample washing and redissolving

1. 70% v/v ethanol

2. Double distilled water

2.6. DNA concentration and purity

UV spectrophotometer

2.7 Conversion to Graphite

1. 4mm internal diameter (i.d.) x 50mm quartz sample tube (special order from Scientific Glass of Florida, Sanford, FL)

2. Copper Oxide (wire form) (Aldrich, Milwaukee, WI)

3. 7mm (i.d.) x 155 mm quartz combustion tube w/breakable tip (Scientific Glass of Florida)

4. 9mm outside diameter (o.d.) x 155 mm borosilicate tube w/dimple 2cm from sealed end (Scientific Glass of Florida)

5. 6mm x 50mm borosilicate culture tube (Kimble/Kontes, Vineland, NJ)

6. Tributyrin (ICN Pharmaceuticals, Costa Mesa, CA)

7. Zinc (powder) (Aldrich)

8. Titanium Hydride (powder) (Aldrich)

9. Cobalt (powder) (Aldrich)

10. Vacuum source (Varian Vacuum Technologies, Lexington, MA)

11. Disposable Vacuum Manifold made from 5/16" plastic y-connector and 1/2" o.d. x

    5/16" i.d. plastic tubing (Nalgene) (See reference 13 for schematic of manifold).

12. Torch for tube sealing (oxy-acetylene type preferred)

13. Muffle Furnace (e.g. NDI Vulcan 3-550)

14. Vacuum Concentrator (e.g. Jouan RC 10.10, Jouan, Winchester, VA)

15. Liquid Nitrogen Bath (consisting of liquid nitrogen in a dewar)

16. Dry Ice/Isopropanol Bath (consisting of a slurry made of dry ice and isopropanol)

3. Methods

3.1   Tissue Homogenization and protein digestion

1. Place 400 mg of fresh tissue in the middle of a piece of plastic wrap and fold the top.

   half of plastic wrap over the bottom half. Cover the wrapped tissue in a layer of

   aluminum foil (see notes 1 and 2).

2. Cover hammer with a plastic bag or plastic wrap. Pound tissue with hammer until

   tissue is well homogenized. Scrape homogenized tissue into a 50 ml tube using a

   clean, disposable scalpel.

3. Add: 25 ml fresh Lysis buffer.

4. Add: 500 μl proteinase K. Mix by vortexing for 5 seconds.

5. Wrap the top of the tubes with parafilm to prevent leakage and contamination.

6. Place tubes in a 37° C shaking water bath overnight, or until tissue appears fully

   digested (i.e. there are no visible lumps of tissue).

7. Centrifuge at 20°C, 2000g, 20 min to remove any undigested tissue.

8. Pour supernatant into a clean 50ml polypropylene tube. Discard pellet.

3.2 RNA digestion

1. Add: 1.25 ml RNAse A to sample.

   1.25 ml RNAse T1 to sample.

2. Mix by vortexing for 5 seconds.

3. Incubate for 30-60 min at room temperature.

3.3 Column purification

1. Add: 1.25 ml 1M MOPS to the sample.

2. Add: 4.5 ml 5M NaCl to the sample.

3. Mix by vortexing for 5 seconds.

4. Stand the required number of Qiagen columns in a rack, with a disposable 50 ml polypropylene tube under each one.

5. To the Qiagen columns, add 25 ml Buffer B.

6. Let the buffer run through the column completely and then discard the buffer.

7. Pour the samples into the columns. Discard the eluate. If column becomes clogged, see note 3.

8. Add: 25 mls Buffer C to each of the columns. Discard the eluate. Repeat and discard the eluate.

9. Add: 25 mls Buffer λ to each of the columns. Collect the eluate in a new, clean polypropylene tube. Keep the eluate, as this will contain the DNA.

3.4   Precipitation of DNA

1. Add 25 ml of ice cold 100% isopropanol to the eluate containing the DNA.

2. Mix by vortexing for 5 seconds.

3. Wrap the lid in parafilm and place in –20ºC freezer overnight to precipitate DNA.

4. Centrifuge at -4°C, 2000g, 3 hours.

5. Carefully pour off supernatant. Save the pelleted DNA, discard the supernatant.

3.5   Sample washing and redissolving

1. Add: 5 mls 70% EtOH to the pellet.

2. Mix by vortexing for 5 seconds.

3. Centrifuge at 4°C, 2000g, 10 min.

4. Save pellet, discard supernatant.

5. Repeat wash step with 70% ethanol and centrifuge.

6. Carefully pour off supernatant, taking care not to dislodge the pellet. If the pellet becomes loose, recentrifuge.

7. Carefully invert the tube on laboratory bench paper to drain excess solvent. Leave tube inverted for 10-15 mins.

8. Add: double distilled water to the pellet (see note 4).

3.6. DNA concentration and purity

1. Dilute an aliquot of the DNA with double distilled water (see note 4).

2. Measure and record the UV absorbance of the diluted DNA at 260nm and 280nm.

3. A 50 μg/ml solution of DNA has a UV absorbance of 1 at 260nm. Therefore, DNA concentration in μg/ml= Absorbance at 260nm x 50 x dilution factor.

4. DNA purity= Absorbance at 260nm/Absorbance at 280nm. Pure DNA should have a ratio of 1.7-1.9 (see note 5).

5. DNA should be prepared for AMS analysis as soon as possible to prevent contamination (see note 6).

3.7 AMS Sample Preparation (Conversion to Graphite)

1. Place all quartz components into muffle furnace and heat to 900°C for two hours. Remove components after they have cooled, handle 6x50mm tubes with disposable forceps only (to prevent contamination).

2. Pipette known amount of DNA into clean, uncontaminated quartz sample tube, add tributyrin if necessary (see note 7). The quartz tube should then be placed within a test tube to protect the sample during vacuum concentration.

3. Remove all volatile components by completely drying with vacuum concentration.

4. Remove quartz sample tube from test tube using disposable forceps. Add 150 – 200 mg of Copper Oxide to dried DNA.

5. Place quartz sample tube in larger quartz combustion tube and evacuate. Seal evacuated combustion tube with torch.

6. Place combustion tube in muffle furnace at 900°C for 2 hours. Remove after it has cooled.

7. Place all borosilicate components into muffle furnace and heat to 500°C for two hours. Remove components after they have cooled, handle 6x50mm tubes with disposable forceps only (to prevent contamination).

8. Place 100 – 150 mg zinc powder and 10-20mg titanium hydride powder into larger borosilicate tube.

9. Place 5-8 mg cobalt powder into 6x50mm borosilicate tube.

10. Drop smaller tube into larger tube so that smaller tube rests on dimple in larger tube, thereby suspending it above the zinc and titanium hydride levels.

11. Place disposable vacuum manifold on vacuum source with the arms of the 'y' hanging down.

12. Push the breakable tip end of the quartz tube into one arm of the 'y' and push the open end of the borosilicate tube onto the other arm.

13. Evacuate the manifold with the tubes attached.

14. Place the quartz tube into the dry ice/isopropanol bath.

15. Keeping the quartz tube in the dry ice/isopropanol bath, place the borosilicate tube into the liquid nitrogen bath.

16. Isolate the manifold from the vacuum source (do not remove from the vacuum source).

17. Crack the breakable tip of the quartz tube allowing the carbon dioxide inside to transfer to the borosilicate tube.

18. Evacuate the manifold again without removing the tubes from either bath.

19. Using the torch, seal off the borosilicate tube above level of the top of the 6x50mm tube trapping the carbon dioxide in the larger borosilicate tube with it's other contents.

20. Place the sealed tube in the furnace and heat to 500°C for 4 hours. Remove after cooling.

21. Break open the larger borosilicate tube to remove the smaller tube. The black powder in the small tube is the graphite, which is analyzed by AMS.

22. AMS will establish the $^{14}C/^{12}C$ ratio of the sample. This is then used to calculate the adduct level (see note 8).

Notes

1. The protocol described is for isolation of up to 400 μg DNA using Qiagen Genomic-tip 500 DNA isolation columns. This is approximately equivalent to the amount of DNA obtained from 400 mg wet tissue. Other column sizes are Qiagen Genomic tip-20 for up to 20μg DNA and tip-100 for up to 90μg DNA. The protocol can be scaled down for the smaller columns (refer to manufacturer instructions for column loading and wash volumes).

2. When handling samples, it is essential to avoid cross-contamination of isotope by the use of disposable plastic ware, scalpels, forceps and gloves. These should be changed in between samples. Liquid samples should be pipetted using filter tips and clean pipettes. When homogenizing the tissue, there should be disposable barriers (plastic wrap and foil) in between the sample and hammer.

3. The flow rate of the column will depend upon the viscosity of the sample. If the column exhibits a very slow flow rate or becomes completely clogged, the flow can be assisted by attaching a small amount of tubing to the bottom of the column and withdrawing eluate slowly with a syringe. Troubleshooting tips are also described in the Qiagen literature.

4. The volume of water required will depend on the amount of DNA extracted. Typically in this procedure, 300 μl of water will result in well dissolved DNA with a concentration of 1-2 mg/ml. A 1:20 dilution of this solution should then be within the range suitable for DNA concentration determination by UV spectrophotometry. The AMS sample tubes used in our laboratory have a sample capacity of about 400 μl, so larger samples may need to be concentrated prior to AMS preparation.

5. If A260nm/A280nm ratios are not within 1.7-1.9, this is probably due to incomplete removal of protein or RNA. The DNA should be repurified prior to analysis by AMS.

6. We try to submit DNA for analysis by AMS within 24 hours of extraction. This will reduce the chance of cross contamination of samples with radioisotope. However, if this is not possible, samples should be stored in a fridge or freezer that is not used for storage of high levels of radioisotope.

7. Tributyrin is a non-volatile hydrocarbon that contains depleted levels of carbon-14. It is used in AMS to increase the size of small samples for efficient graphitization. Typically using this method, samples that contain less than 0.5 mg carbon require the addition of carrier. For DNA (29% Carbon), this would be equal to 1.7 mg. A 40 mg/ml solution of tributyrin in methanol is made and then 50μl added to each DNA sample. Carrier controls should be prepared at the same time (see note 8).

8. The isotope ratios determined by AMS are converted to adduct levels firstly by subtracting the natural radiocarbon content of the sample and the radiocarbon contributed from addition of any carrier. The natural radiocarbon content of the DNA is determined using control DNA samples from subjects or rodents not administered the $[^{14}C]$-labeled compound. Adduct levels (ratio of moles of compound:moles nucleotide) are then calculated based upon the % carbon of DNA (29%) and the compound specific activity (16).

Acknowledgements

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APPENDIX 2

Effect Of Dietary Constituents With Chemopreventive Potential On Adduct Formation Of A Low-Dose Of The Heterocyclic Amines PhIP And IQ And Phase II Hepatic Enzymes

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Abstract:

We conducted a study to evaluate dietary chemopreventive strategies to reduce genotoxic effects of the carcinogens 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). PhIP and IQ are heterocyclic amines (HCAs) that are found in cooked meat and may be risk factors for cancer. Typical chemoprevention studies have employed carcinogen doses many thousand-fold higher than usual human daily intake. Therefore, we administered a low dose of $[^{14}C]$PhIP and $[^3H]$IQ and utilized accelerator mass spectrometry to quantify PhIP-adducts in the liver, colon, prostate and blood plasma and IQ adducts in the liver and blood plasma with high sensitivity. Diets supplemented with phenethylisothiocyanate (PEITC), genistein, chlorophyllin or lycopene were evaluated for their ability to decrease adduct formation of $[^{14}C]$PhIP and $[^3H]$IQ in rats. We also examined the effect of treatments on the activity of the phase II detoxification enzymes glutathione-*S*-transferase (GST), UDP-glucuronyltransferase (UGT), phenol sulfotransferase (SULT) and quinone reductase (QR). PEITC and chlorophyllin significantly decreased PhIP-DNA adduct levels in all tissues examined, which was reflected by similar changes in PhIP binding to albumin in the blood. In contrast, genistein and lycopene tended to increase PhIP adduct levels. The treatments did not significantly alter the level of IQ-DNA or -protein adducts in the liver. With the exception of lycopene, the treatments had some effect on the activity of one or more hepatic phase II detoxification enzymes. We conclude that PEITC and chlorophyllin are protective of PhIP-induced genotoxicity following a low exposure dose of carcinogen, possibly through modification of HCA metabolism.

Abbreviations:

AMS, Accelerator mass spectrometry; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium salt; GSH, reduced glutathione; GST, glutathione-*S*-transferase; HCA, heterocyclic amine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PEITC, phenylethylisothiocyanate; PhIP, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine; PNP, p-nitrophenol; QR, quinone reductase; SULT, phenol sulfotransferase; UGT, UDP-glucuronosyl transferase.

Introduction:

It is generally believed that a significant proportion of human cancer is caused by dietary factors, such as exposure to the heterocyclic amines (HCAs) 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). PhIP and IQ are compounds that occur in well-cooked meats and fish (1-5) and are mutagens in mammalian cells (6,7). Importantly, PhIP and IQ produce tumors at multiple organ sites in rodent models. PhIP causes breast, colon and prostate tumors in rats (8-9), whereas IQ causes tumors mainly in the liver and small and large intestines (10). The role of PhIP and IQ in human cancer is further supported by some studies that show a positive correlation between meat consumption and the incidence of cancer (11-17).

The mutagenicity, and presumably the carcinogenicity, of PhIP and IQ result from metabolic activation of the parent compounds to a form that will bind to DNA, forming DNA adducts (18). DNA adduction is important since it is thought to be an initiating event in chemical carcinogenesis (19). Consequently, DNA adducts should be useful in assessing the efficacy of agents in preventing a carcinogen from initiating cancer. Likewise, protein adducts provide a measure of metabolic activation capability and may be a useful surrogate for DNA damage in the tissues (20). Therefore, adducts with tissue proteins such as plasma albumin may assist in the monitoring of populations for changes in cancer risk, which may be achieved through exposure avoidance and chemoprevention (21).

The long-term goal of our work is to establish chemopreventive strategies for HCA-induced cancer. However, studies to determine the efficacy of chemopreventive agents in reducing cancer risk following carcinogen exposure typically involve the administration of large doses of carcinogen (mg/kg body-weight), which are often several orders of magnitude higher than the typical human daily intake. Carcinogen metabolism and adduct formation can be greatly affected by the exposure dose of a carcinogen (22). Therefore, the ability to conduct chemoprevention studies using

carcinogen doses that are more representative of dietary levels of exposure is crucial in the design of effective preventive interventions. In this study, we used the highly sensitive method of accelerator mass spectrometry (AMS) to examine the effect of chemopreventives on adduct levels following a simultaneous low exposure dose of $[^{14}C]$PhIP and $[^{3}H]$IQ (approximately 2 μg of each HCA/animal). Human exposure estimates for the HCAs vary widely, but up to 480 ng PhIP/g has been reported in very well cooked chicken (1) and 20 ng IQ/g has been detected in broiled sardine (23). Based upon a dose/kg body-weight comparison, and an average 70 kg adult human consuming 100g of very well-done chicken or sardine, the rats received approximately a 12-fold higher dose of PhIP and a 372-fold higher dose of IQ than would be expected from consumption of a single meal. Although the doses are not within dietary-exposure levels, they are several thousand-fold lower than many other studies published to date.

We tested phenylethylisothiocyanate (PEITC), chlorophyllin, lycopene and genistein for their ability to reduce PhIP-DNA adduct formation in colon and prostate (target organs for PhIP-induced carcinogenicity) and the liver (the major xenobiotic-metabolizing organ) (8,9), as well as IQ-DNA adduct levels in the liver (a target organ for IQ) (10). The chemopreventives were chosen because several epidemiology and laboratory animal studies have indicated that they may be protective against cancer (24-27). Some evidence has suggested that these compounds decrease the risk of chemically induced cancers by reducing the active carcinogen level in the tissues, thus reducing adduct formation (28-31). However, the ability of these chemopreventives to reduce adduct levels following low carcinogen exposures is not known. The chemopreventive compounds were consumed as an integral part of a nutritionally adequate amino acid-based diet in this study. We selected the amino acid-based diet because it is free of the test compounds (before supplementation), its composition can be controlled and monitored reliably, and it promotes growth in rats that is equivalent to that of a casein-based diet (32). In order to establish potential mechanisms of action of the chemopreventives, we also investigated the effect of the treatments on

the amounts of the HCAs present in tissues and the activity of several enzymes that may play an important role in carcinogen detoxification and cancer susceptibility (reviewed in 33).

Materials and methods

Chemicals:

PEITC was purchased from LKT Laboratories (St. Paul, MN). Genistein was purchased from Indofine Chemical Company (NJ). Lycopene (as LycoVit) was obtained from BASF (Ballerup, Denmark) as a 10% w/w powder. Chlorophyllin (sodium-copper salt) was obtained from Sigma Chemical Co. (St. Louis, MO). The radio-labeled carcinogen, [^{14}C]PhIP, specific activity 61 mCi/mmol, was from Toronto Research Chemicals (Ontario, Canada), and was purified by HPLC to a chemical and isotopic purity of >99%. The radio-labeled carcinogen, [^{3}H]IQ, 2200 mCi/mmol was from Chemsyn Science Laboratories (Lenexa, KS) and was purified by HPLC to >99% chemical and radiopurity. It was then diluted in unlabeled IQ to a specific activity of 133 mCi/mmol prior to use. The dilution was made in order to obtain similar chemical doses for both PhIP and IQ. Solvable tissue solubilizer was from NEN Research Products (Boston, MA) and Hionic-Fluor NCSII scintillation cocktail (used with solubilized samples) was obtained from Packard Instrument Company (Meriden, CT). Affi-gel blue gel and dye reagent for protein assays was from Bio Rad (Richmond, CA). Tributyrin was obtained from ICN Pharmaceuticals, Inc, (Costa Mesa, Ca). All other chemicals were of analytical grade and purchased from Sigma-Aldrich.

Animals and diets:

The study was approved by the Institutional Animal Care and Use Committee at Lawrence Livermore National Laboratory in accordance with the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male rats (5-6 week old F344 strain) were purchased from Simonsen Laboratories (Gilroy, CA). After a quarantine period of 7 days, they were transferred to a nutritionally adequate amino acid based diet (32) for a six day adjustment period.

The animals were then systematically divided into groups (5 animals per group) which exhibited equal mean growth rates (as assessed by body-weight gain per day). The groups were randomly assigned to the treatments. For the next 15 days, they were given *ad libitum* access to diets that had been fortified with PEITC, genistein, chlorophyllin, or lycopene. Two groups of animals received no fortificant and acted as controls.

The doses of chemopreventive were greater than human daily exposures, but were chosen based on a survey of literature utilizing these compounds in animal models of chemoprevention. The doses were as follows: 816 mg PEITC/kg diet (34-35), 500 mg genistein/kg diet (36-37), 10 g chlorophyllin/kg diet (38-39) or 10 g Lycovit/kg diet (equivalent to 1 g lycopene/kg diet) (40). For reference, some estimated human daily exposures for the chemopreventive compounds are as follows: 9.1 μmol total isothiocyanates (41), equivalent to 1.5 mg of PEITC if the only source of iosthiocyanates was PEITC; the average intake of isoflavones, such as genistein, from foods by Asian populations is estimated at 15-50 mg per day (42-43), whereas current U.S. average intake is much less (44); a person would consume approximately 0.13-0.18 mg chlorophyll from 100g of spinach or kale (45); 25.2 mg lycopene per person per day (46).

PhIP dosing:
On the 14th day, rats were dosed once with [^{14}C]PhIP and [^{3}H]IQ by gavage. We chose to co-administer the HCAs in this study to more closely reflect the multiple exposures arising from the consumption of HCA-containing food. The [^{14}C]PhIP and [^{3}H]IQ were dissolved in ethanol and added to corn oil to a concentration of 1% v/v ethanol. Each rat received 4 μl/g body weight (8.1 μg [^{14}C]PhIP/kg body-weight and 10.8 μg [^{3}H]IQ/kg body-weight). For reference, the average weight of the rats prior to PhIP dosing was 241g, hence the average dose of [^{14}C]PhIP or [^{3}H]IQ was 2 μg of each per animal. One group of control rats received corn oil containing no [^{14}C]PhIP or [^{3}H]IQ, which provided a value for the background level of ^{14}C and ^{3}H in samples.

Tissue collection and storage:

Twenty-four hours after dosing (day 15), each rat was euthanized by CO_2 asphyxiation. Blood was collected by cardiac puncture into tubes containing heparin, as anticoagulant, and centrifuged at 3000xg for 10 minutes, to separate the plasma. Liver, colon and prostate (ventral, dorsolateral and anterior prostate) were excised promptly. Intestinal contents were flushed from the colons using 5-10ml of phosphate buffered saline. Plasma, liver, colon and prostate were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Extraction of DNA:

DNA was extracted from tissue using Qiagen DNA isolation columns (Qiagen Inc., Valencia, CA) using previously published methods (47). The extracted DNA was re-dissolved in 300 µl distilled deionized water. The concentration of DNA was calculated by measuring the absorbance at 260 nm (A260 nm), assuming an absorbance value of 1.0 is equal to a DNA concentration of 50 µg/ml. DNA purity was determined from the A260 nm/A280 nm ratio. All DNA used in this study had a ratio of 1.6-1.8.

Extraction of proteins:

Blood albumin:

Approximately 0.5-1.0 ml plasma was dialyzed for 2 days against 2 changes of 250 ml of 0.1 M potassium chloride, 0.05 M Tris, pH 7.0 to remove unbound [^{14}C]PhIP and [^{3}H]IQ. Albumin was then purified from dialyzed plasma by affinity chromatography on Affi-Gel Blue Gel columns using previously published methods (48). The albumin solution was then concentrated to approximately 1 ml using Centricon Plus filter units (Millipore) and dialyzed for 2 days against 2 changes of double distilled water to remove salt, which can interfere with AMS analysis. Albumin concentrations were determined using Bio-Rad protein assay reagent and bovine serum albumin standards, as described by the manufacturers. The albumin samples were analyzed by AMS for ^{14}C and ^{3}H content, as described below.

Liver protein:

To extract protein from the liver tissue samples, 500 mg aliquots of tissue were homogenized, and added to tubes containing 5 ml lysis buffer (4 M urea, 1% (v/v) Triton X-100, 10 mM EDTA, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 10 mM dithiothreitol). Samples were placed in a 37°C shaking water bath over-night. To remove any incompletely lysed tissue, samples were centrifuged at 3000xg for 15 minutes. 500 μl of 70% (v/v) perchloric acid was added to the supernatant to precipitate protein. Samples were vortexed, and centrifuged at 3000xg for 15 minutes. The resulting pellets were then washed with 5% (v/v) perchloric acid, twice with 50% (v/v) methanol, and twice with 1:1 (v/v) ether:ethanol. Pellets were then air dried. This methodology would not liberate pure protein, as the extracts will contain other acid-precipitable macromolecules.

Analysis of ^{14}C and ^{3}H content of plasma and liver tissue: Plasma and liver samples were analyzed by liquid scintillation counting to determine the amount of [^{14}C]PhIP and [^{3}H]IQ and their metabolites present 24 hours post-exposure. This was performed in order to investigate if any of the chemopreventives altered HA bioavailability in the tissues. For example, chlorophyllin has been shown to decrease HA absorption from the intestine (49). One hundred microliters of plasma was added to 20 mL of scintillation fluid and counted for 10 min each using a Pharmacia Wallac 1410 liquid scintillation counter (Gaithersburg, MD) and the resulting dpm were converted to ng HCA/ml plasma.

Two ml of tissue solubilizer was added to vials containing 91-135 mg liver tissue and the samples were left in a shaking waterbath at 30°C for 12 h. After solubilization was complete, 10 mL of Hionic-Fluor NCSII scintillation cocktail was added and the samples were counted, as above.

AMS Analysis:

A portion of each DNA or albumin solution (315-484 μg colon or liver DNA, 22-85 μg prostate DNA or 69-1000 μg albumin from each animal) was added to quartz tubes with 2 mg tributyrin

added as a carrier to provide sufficient total carbon or hydrogen for AMS. Approximately 5-10 mg of liver protein from each animal was analyzed without the addition of carrier. Due to sample size limitations, the [^{3}H]IQ content of colon or prostate DNA was not analyzed in this study. Samples were prepared for AMS analysis and the ^{14}C/^{13}C and ^{3}H/^{1}H ratios measured as previously described (50-52). The ratios were converted to adduct levels following the subtraction of the ^{14}C and ^{3}H contribution from any added tributyrin and control samples.

AMS measures only the amount of ^{14}C and ^{3}H in a sample as an isotope ratio and provides no structural information, and thus no distinction between HCA metabolites, adducts and parent compound within a sample can be made.

Assay of Phase II liver enzymes:

1. Glutathione-*S*-transferase:

Frozen rat hepatic tissues were thawed on ice. Tissues were homogenized (1 g tissue/2 ml buffer) in a 10 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose, 1 mM DTT, and 1 mM EDTA, as described previously (53). The homogenate was centrifuged at 10,000xg for 30 minutes. The supernatant was collected and centrifuged at 100,000 g and 4°C for 80 minutes to remove microsomal GST, leaving cytosolic forms of GSTs in the supernatant.

GST activity was measured using GSH and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (54). Activity of the enzyme was determined in a 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM GSH and 1 mM CDNB using an extinction coefficient of 9.6 mM^{-1} cm^{-1} at 340 nm for CDNB or CDNM-GSH conjugate. One unit of enzyme is defined as the amount required to catalyze the conjugation of 1 μmol of substrate to GSH per minute at 25°C. Protein concentrations were determined using bovine serum albumin as a reference. GST activity is reported as activity per unit cytosolic protein.

2. Quinone reductase:

QR activity was determined according to Prochaska and Santamaria, 1988 (55) by following the reduction of menadione to menadiol in the presence of MTT and a NADPH-generating system. The associated nonenzymatic reduction of MTT by menadiol to the blue formazan was recorded at 610 nm using a Beckman DU-600 spectrophotometer in kinetic mode. Enzyme reactions were started by the addition of menadione and were carried out in a total volume of 1 ml at 25°C under conditions of demonstrated linearity with respect to time and protein amount. Typically, 200 μg protein extract (100,000xg supernatant) was used per reaction. The specific activity of quinone reductase (nmol min^{-1} mg^{-1} protein) was calculated based on the extinction coefficient of reduced MTT (11.3 mM^{-1} cm^{-1} at 610 nm).

3. Sulfotransferase:

The activity of phenol sulfotransferase was assayed using a colorimetric assay in which the enzyme catalyzes the synthesis of 2-naphthylsulfate from 2-naphthol and 5'-phosphoadenosine 3'-phosphosulfate (PAPS), as previously described in Frame *et al.*, 2000 (56), with modifications (57).

4. UDP-glucuronosyltransferase:

An initial screen for activity of the UGT1A family of UGT was performed using p-nitrophenol as the substrate on pooled samples from each of the treatments. This was performed according to Irshaid *et al.*, 1987 (58). N-hydroxy-PhIP (NOH-PhIP) glucuronidation rates were then determined on each individual sample as described by Nowell *et al.*, 1999 (59), with the addition of 15 mg/ml bovine serum albumin in the incubation mixture.

Data analysis:

AMS data represent the mean ± the standard deviation of 5 replicate animals. The effects of the supplements were analyzed with ANOVA and differences among means evaluted with Fisher's

PLSD using Statview software Version 5.0.1 (Abacus Concepts, Inc.).   Statistical significance of differences between means of the groups was defined as a P value ≤ 0.05.

Results:

The animals grew well (average weight gain was 4.6 ± 0.3 g/day) and remained healthy throughout the study.   There were no significant differences in mean body-weights and growth rates between the controls and the animals receiving chemopreventive-supplemented diets (**Table 1**).

The effect of the diets on PhIP-DNA adduct levels in the liver, colon, and prostate and IQ-DNA adduct levels in the liver 24 hours following HCA exposure are shown in **Figure 1**. In all animals, the highest DNA adduct level was with IQ in the liver, followed by PhIP in the prostate>colon>liver. PhIP-DNA adduct levels in the PEITC-treated animals were 1.2 to 1.7-fold lower in all organs examined and 1.5 to 1.6-fold lower in chlorophyllin-treated animals. Genistein significantly increased PhIP-DNA adduct levels in the colon and prostate (by 1.2-fold in each) and lycopene increased PhIP-DNA adduct levels in the liver by 1.5-fold. None of the treatments significantly affected IQ-DNA adduct levels.

Adduct formation with liver protein and plasma albumin is shown in **Figure 2**. Protein adduct formation was influenced by all the treatments in either the liver, plasma or both. PhIP-protein adduct formation with plasma albumin was significantly reduced by PEITC (1.5-fold) and chlorophyllin (1.3-fold). However, genistein and lycopene generally had the opposite effect. Genistein increased PhIP and IQ adduct levels with plasma albumin (1.3 to 1.4-fold) and PhIP-protein adduct formation in the liver (1.3-fold). Lycopene treatment resulted in significantly higher IQ-albumin (1.6-fold) and PhIP-liver protein adduct levels (1.2 fold).

The effect of the diets on the amount of $[^{14}C]PhIP$ and $[^{3}H]IQ$ and their metabolites detected in the plasma and liver tissue are summarized in **Table 2**. The amount of $[^{14}C]PhIP$ and $[^{3}H]IQ$ measured

in these tissues 24 hours post-exposure were affected by supplementation with genistein and PEITC. Genistein increased the amount of radio-labeled PhIP by 1.6 fold in the liver tissue and slightly increased the amount of IQ detected in the plasma. PEITC increased the amount of PhIP in the liver by 1.5-fold.

The results of the enzyme assays conducted on liver extracts are shown in **Table 3**. PEITC had the greatest effect and increased GST and QR 1.5-1.8 fold over the controls. PEITC also increased UGT activity as measured using PNP as the substrate, however no effect was seen with the bioactive PhIP metabolite, NOH-PhIP. It had the opposite effect on SULT and decreased activity by 1.1 fold. Genistein treatment also led to a slight decrease in SULT activity and increase in UGT, using PNP as a substrate. Chlorophyllin treatment only affected QR and led to a 1.6 fold increase in activity. Lycopene had no significant effect on the enzymes examined.

Discussion:

Toward the goal of developing effective cancer chemoprevention strategies, various dietary agents have been investigated for their ability to alter carcinogen metabolism and reduce adduct formation (reviewed in 60-61). However, a major limitation of these studies is that they have been performed using very high doses of carcinogens, typically several orders of magnitude greater than human exposures. The use of such high doses was necessary because of the lack of sensitive methodology for measuring carcinogen metabolites or adducts at lower doses relevant to human consumption. Thus, by using radio-labeled carcinogens and the very sensitive AMS technique, we demonstrate the feasibility of investigating a much lower exposure of carcinogen *in vivo*.

Exposure to PhIP and IQ at high doses has been shown to result in DNA adduct formation in the liver and several other extrahepatic organs, including the prostate and colon in rats (9, 62-64). In this study, the highest DNA adduct level was seen by IQ in the liver, followed by PhIP in the prostate>colon>liver. These results are consistent with previous data showing that the liver is a

target organ for IQ-induced carcinogenicity and the prostate and colon are targets for PhIP-induced carcinogenicity, whereas the liver is not (8-10).

Our data demonstrate that PEITC and chlorophyllin significantly reduce DNA adduct levels in the target organs for PhIP and may, therefore, be useful in preventing cancer initiation. However, genistein and lycopene actually increased PhIP-DNA adduct levels in some organs, which may indicate that they increase cancer initiation. Although the changes in adduct levels were modest (1.2 to 1.7-fold), this study was conducted with only a single dose of HCA. Following chronic exposure to carcinogens, the effects of chemopreventives on adduct levels may be much greater. Future studies are planned to establish the effect of PEITC and chlorophyllin in a long-term PhIP feeding study in rats to establish if the agents reduce tumor incidence, as well as adduct levels.

The ability of chemopreventive agents to reduce tumor formation following carcinogen exposure has been partially attributed to their ability to reduce DNA adduct levels in target tissues, either through altering the bioavailable dose or altering carcinogen metabolism (65-66). Conjugation of the bioactivated carcinogen to glutathione by GST is considered an important detoxification pathway for PhIP, but not IQ (67-68). Sulfation catalyzed by SULT may be both a detoxification and an activation pathway for PhIP (69-70). Glucuronidation is an important detoxification pathway for HCAs in rats and humans (71) and the UGT1A subfamily has specifically been shown to be important in NOH-PhIP detoxification (59). Therefore, as these enzymes are important in the metabolism of HCAs, they could mechanistically be involved in the chemoprevention of HCA-induced cancer.

Some evidence has shown that natural isothiocyanates target mammalian Phase I and Phase II drug-metabolizing enzymes and their coding genes, resulting in decreased carcinogen-DNA interactions and in increased carcinogen detoxification (72). One naturally occurring isothiocyanate is PEITC. In this study, we found that PEITC treatment increased PhIP levels in the liver (which could be due

to parent compound, metabolites or adducts). It also increased the activity of the phase II detoxification enzymes GST, QR and UGT using PNP as a substrate. This would indicate that a reduction in adduct levels may have occurred through induction of one or more of these enzyme pathways. This may have led to accumulation of detoxification products in the liver tissue. However, PEITC had no significant effect on UGT activity, as measured using NOH-PhIP as the substrate. The reason for the discrepency is possibly that PNP is a major substrate for UGT1A6, which is highly expressed in the liver and does not glucuronidate NOH-PhIP (73). Therefore, the effect on UGT may only be specific for isozymes that are not involved in HCA metabolism. PEITC also slightly reduced SULT activity and this may result in a reduction in adduct levels, since SULT may be involved in HCA bioactivation to a DNA-binding species (74-75).

The chemopreventive effect of PEITC is consistent with the observations of others where PEITC reduced HCA mutagenicity in bacterial assays (76-77). However, this effect of PEITC is inconsistent with the observation that a single dose of PEITC did not reduce adduct levels in mice treated with a high dose of [^{14}C]PhIP (175 mg/kg body-weight) (76). The differences in rodent species studied may account for this inconsistency. It is also possible that because our rats were fed the PEITC-supplemented diet for 14 days prior to PhIP dosing, their tissue PEITC had stabilized at an optimum level and provided sufficient time for enzyme induction to occur. Most importantly, we used a 17,500-fold lower dose of PhIP. Knasmuller *et al.*, 1996 (76) hypothesized that the capacity of mammalian cells to further activate NOH-PhIP to reactive metabolites is probably a limiting step in PhIP bioactivation to a DNA binding species. Thus, the more physiologically relevant PhIP dose utilized in this study allowed for the protective effect of PEITC to be evident.

Chlorophyllin also exhibited chemopreventive effects against PhIP-DNA adducts in our study, but in contrast to other published studies (38), it did not significantly reduce IQ-DNA adduct levels. Chlorophyllin may act through several mechanisms. It has been shown to reduce HCA

bioavailability in tissues through complex formation and prevention of absorption from the digestive tract and increases phase II detoxification reactions (78). We did not observe a reduction in the amount of HCA in the plasma or liver following exposure, although we only analyzed samples 24 hours post-exposure when most of the compounds are likely to have cleared the body. However, we did see an induction of QR in the liver. Although this enzyme is not considered a detoxification pathway for HCAs, it is a convenient method for screening of Phase II enzyme induction, as it is induced coordinately with other electrophile-processing Phase II enzymes (55). Therefore, although we did not see a change in activity of GST, SULT or UGT, a PhIP-detoxification pathway may have been induced. We do not know why PhIP-DNA adducts would be reduced by chlorophyllin treatment, whilst IQ-DNA adducts remain unchanged, but it is possible that due to the larger variation in the data from the analysis of ^{3}H by AMS, we were not able to detect a small change in IQ adduct levels.

Genistein was not chemopreventive against DNA adduct levels in our study, as it actually increased PhIP-DNA adduct levels in the colon and prostate. Soymilk containing genistein has been shown to reduce mammary tumor incidence *in vivo* in rats following exposure to PhIP (79), which implies that genistein may act through some other mechanism, such as through protection against PhIP-induced oxidative stress (80) or via prevention of tumor promotion or progression. Interestingly, genistein treatment also resulted in slightly increased levels of HCA in the liver and plasma and higher protein adduct levels in these tissues. It also caused decreased SULT activity and increased UGT activity. However, the increase in UGT activity was not towards NOH-PhIP and may not effect PhIP adduct levels. SULT is also a detoxification pathway for PhIP, so it is possible that this diverted a bioactive PhIP metabolite towards adduct formation.

Lycopene treatment increased the PhIP-DNA adduct level in the liver, but did not alter the amount of HCAs measured in the tissues or the levels of phase II hepatic enzymes. It slightly increased the PhIP-liver protein adduct level and the IQ-albumin adduct level in the plasma. This data implies that

lycopene may not be chemopreventive for HCA-DNA adducts and is consistent with studies that show lycopene does not alter tumor incidence in rats following treatment with high doses of PhIP (81).

We have previously shown that PhIP and other HCAs form adducts with proteins in animals and humans and that adduct levels were dose-dependent (48, 82-83). Therefore, this data indicated that blood protein adducts and protein adducts in target tissues may provide a measure of the bioactive dose in the tissues. In this current study, PhIP-albumin adduct levels were significantly reduced by PEITC and chlorophyllin and significantly increased by genistein. Importantly, as these compounds similarly altered DNA adduct levels in the target organs for PhIP-induced tumors, this would imply that measuring albumin adduct levels may be useful for screening the ability of chemopreventive agents to reduce the bioactive dose of a carcinogen in tissues. However, this relationship was not as clear for IQ, as we observed an increase in IQ-albumin adduct levels with genistein and lycopene, but a similar effect was not detected in corresponding DNA adduct levels. This may be because any differences in IQ-DNA adduct levels between treatments were too small to be detected within the analysis errors of this study.

Tissue proteins are often analyzed as a potential biomarker of DNA adducts because they tend to have higher levels of binding and are present in larger quantities in the tissues, making them a more easily measurable marker than DNA (82). However, measurement of HCA binding to a relatively impure mixture of proteins in a tissue may not provide reliable information about DNA adduct levels in the same organ. For example, a reduction in PhIP-liver protein adduct levels was not detected with PEITC and chlorophyllin, although these treatments significantly reduced DNA adduct levels in the liver. Better correlations may have been achieved if purified proteins were analyzed.

In summary, our protocol using a well-defined diet and low doses of radioisotope-labeled HCA enabled us to observe significant changes in response to chemoprotective dietary supplements in a rodent model. We have shown that the isothiocyanate PEITC and chlorophyllin significantly reduce DNA adduct formation using a low-dose of PhIP in rats, possibly through the modification of PhIP metabolism. Therefore, this finding would suggest that these compounds may protect against PhIP-induced carcinogenicity following dietary-relevant carcinogen exposures. Future work will focus on testing the ability of lower doses of PEITC and chlorophyllin in reducing PhIP genotoxicity.

Acknowledgments and Notes:

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	None (control)	PEITC	Genistein	Chlorophyllin	Lycopene
Number of Rats/treatment	5	5	5	5	5
Initial weight, g	121 ± 5	121 ± 10	127 ± 9	121 ± 12	115 ± 5
Growth rate, g/d	4.6 ± 0.2	4.6 ± 0.3	4.4 ± 0.3	4.6 ± 0.7	4.5 ± 0.2
Final weight, g	241 ± 7	243 ± 7	236 ± 10	247 ± 18	238 ±13

Table 1. Weights and growth rates of rats fed an amino acid based diet supplemented with chemopreventives. Data are presented as means ± standard deviation.

Tissue	HCA	None (control)	PEITC	Genistein	Chlorophyllin	Lycopene
Liver	PhIP	0.65 ± 0.20	0.95 ± 0.23* **1.5 fold** ↑	1.03 ± 0.26* **1.6 fold** ↑	0.59 ± 0.12	0.85 ± 0.13
Liver	IQ	7.26 ± 0.81	7.38 ± 0.68	7.82 ± 0.60	7.76 ± 0.54	7.21 ± 0.31
Plasma	PhIP	0.19 ± 0.05	0.18 ± 0.06	0.21 ± 0.06	0.14 ± 0.04	0.18 ± 0.03
Plasma	IQ	5.69 ± 0.34	5.70 ± 0.34	6.10 ± 0.1* **1.1 fold** ↑	5.86 ± 0.21	5.82 ± 0.39

Table 2. Liver tissue and plasma concentrations of [^{14}C]PhIP and [^{3}H]IQ and their metabolites following administration of [^{14}C]PhIP and [^{3}H]IQ to rats fed an amino acid based diet supplemented with chemopreventives. Units are ng HCA/g tissue or ml of plasma. Data are presented as means ± standard deviation. *Data are statistically different from the control treatment (P≤0.05).

	None (control)	PEITC	Genistein	Chlorophyllin	Lycopene
GST	0.280 ± 0.032	0.407 ± 0.053* **1.5 fold** ↑	0.279 ± 0.050	0.316 ± 0.040	0.282 ± 0.033
QR	13.78 ± 2.20	25.22 ± 3.13* **1.8 fold** ↑	16.96 ± 3.38	21.40 ± 5.59* **1.6 fold** ↑	17.24 ± 2.27
SULT	11.81 ± 1.18	10.29 ± 0.75* **1.1 fold** ↓	9.56 ± 0.71* **1.2 fold** ↓	12.50 ± 0.94	11.74 ± 0.62
UGT (PNP as substrate)	17.53 ± 0.47	21.88 ± 1.99* **1.2 fold** ↑	22.50 ± 2.26* **1.3 fold** ↑	16.86 ± 1.65	18.81 ± 0.45
UGT (NOH-PhIP as substrate)	865 ± 96	879 ± 91	779 ± 95	805 ± 62	795 ± 65

Table 3. Enzyme specific activities in the liver of rats fed an amino acid based diet supplemented with chemopreventives. Units for the GST and QR assays are μmol product formed per mg protein in the 100,000xg supernatant extract fraction. SULT units are nmol product/min/mg protein and UGT units are pmol/min/mg protein. Data are presented as means ± standard deviation of samples from individual animals in each treatment group, except for the UGT assay with PNP, in which samples within each treatment group were pooled for analysis and the assay repeated 3 times. *Data are statistically different from the control treatment (P≤0.05).

Figure Legends:

Figure 1. PhIP-DNA adduct levels in tissues following administration of [^{14}C]PhIP and [^{3}H]IQ to rats fed an amino acid based diet supplemented with chemopreventives. Data are presented as means ± standard deviation. *Data are statistically different from the control treatment (P≤0.05).

Figure 2. PhIP-protein adduct levels with liver protein and plasma albumin following administration of [^{14}C]PhIP and [^{3}H]IQ to rats fed an amino acid based diet supplemented with chemopreventives. Data are presented as means ± standard deviation. *Data are statistically different from the control treatment (P≤0.05).

Figure 1.

Figure 2.

Legend:
- Albumin (PhIP)
- Albumin (IQ)
- Liver protein (PhIP)
- Liver protein (IQ)

y-axis: pg HCA/g protein (10^4, 1000)

x-axis (Treatment): Control, PEITC, Genistein, Chlorophyllin, Lycopene
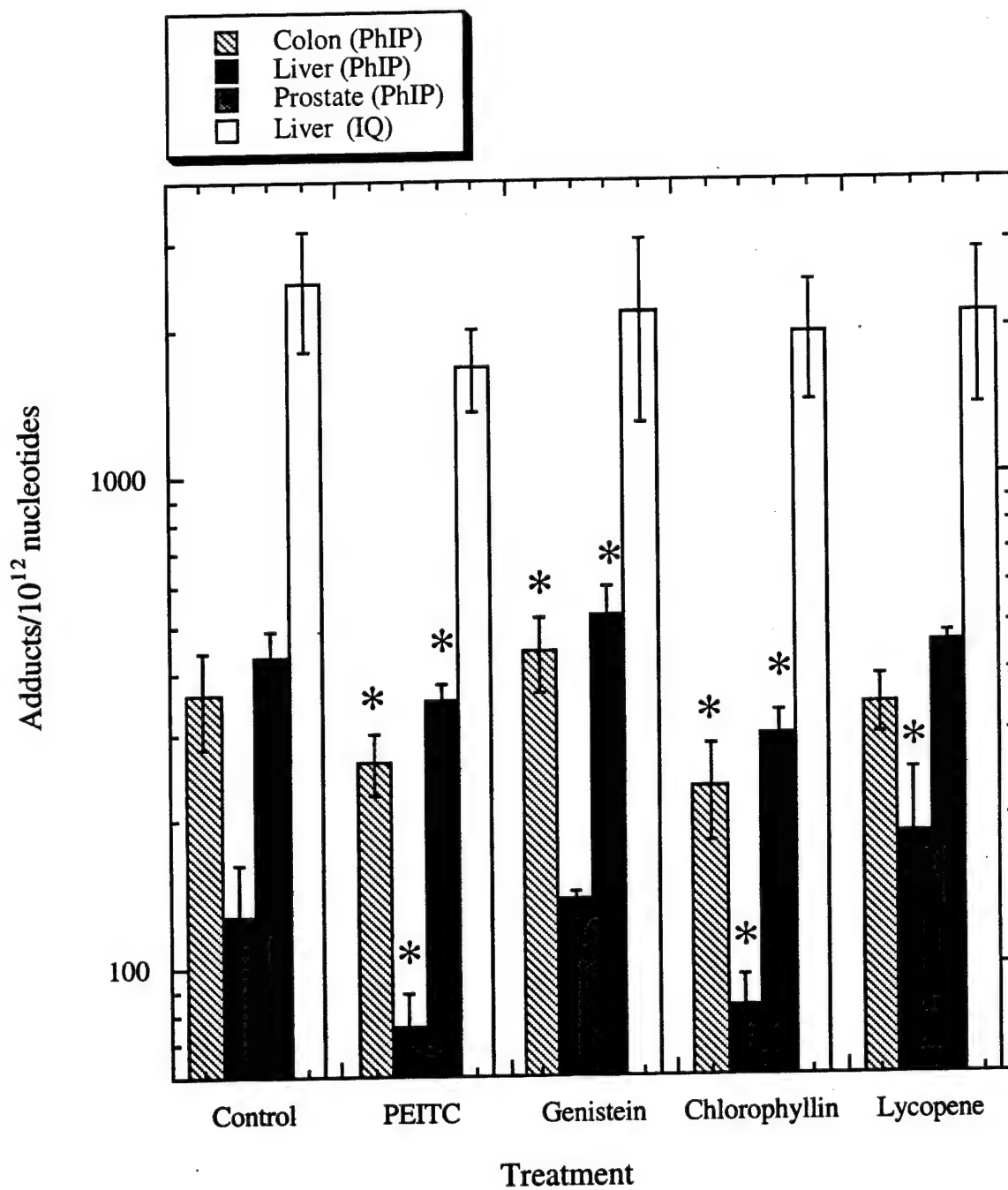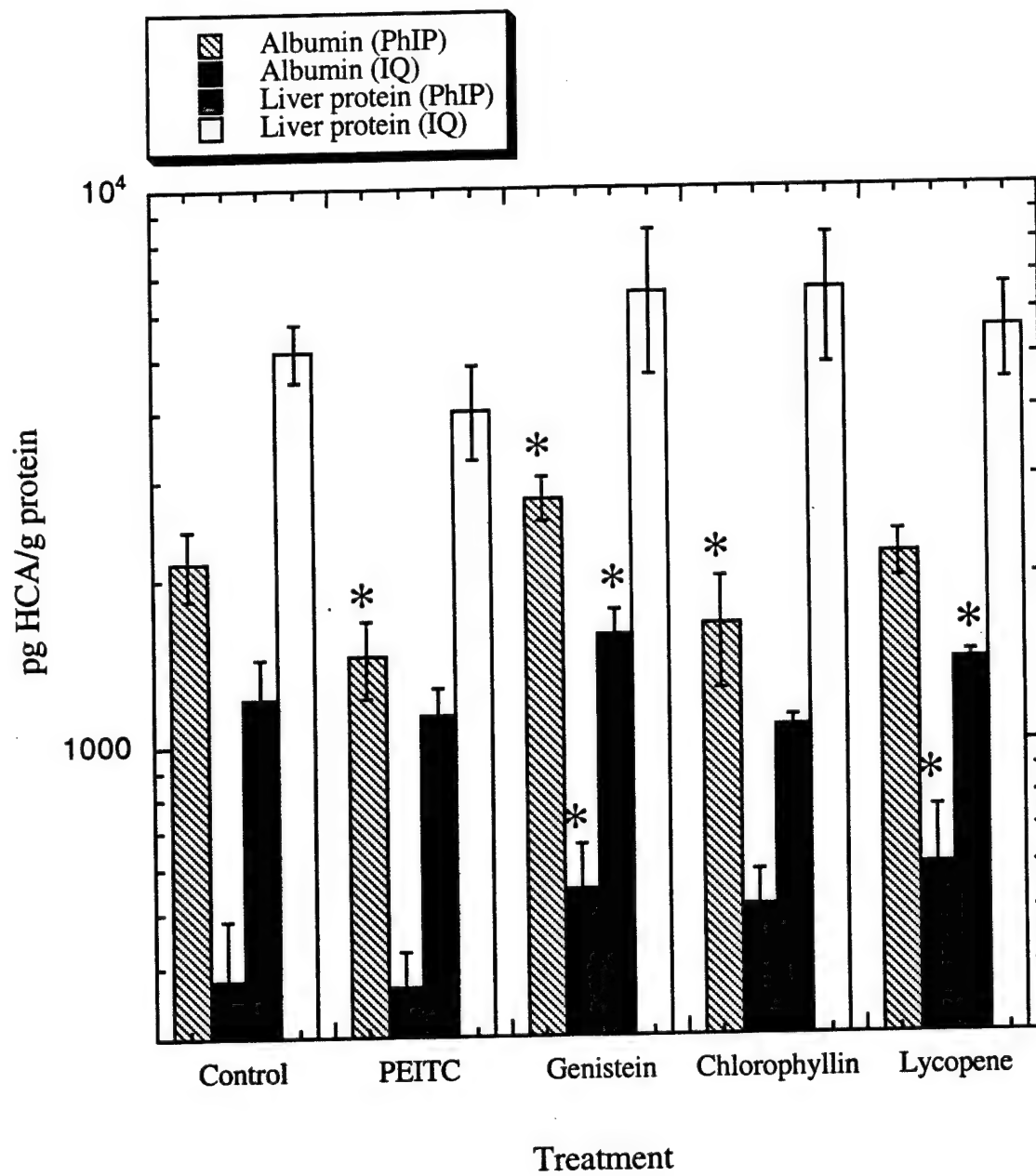
ANIMAL MODELS AND ANALYTICAL APPROACHES FOR UNDERSTANDING THE RELATIONSHIPS BETWEEN WINE AND CANCER

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INTRODUCTION

Epidemiologic studies have shown that moderate wine consumption may be linked to a decreased risk of many degenerative diseases, including cancer (Grønbaek et al., 1995, 2000). However, establishing the exact nature of the bioactive compounds and their mechanism of action have proven elusive. This is in part due to the varying protocols to study the chemopreventive effects of dietary components. Animal models that use high doses of carcinogens to induce tumors can result in unpredicatable kinetics of tumor formation as well as undefined changes in the metabolism of dietary components. Administration of test compounds at non-physiologic levels (e.g., by gavage or injection) can lead to variable effects on biologic availability or efficacy as compared to normal dietary levels when consumed orally. And finally, the nutritional status of the test animals has not always been well-defined or controlled.

Our approaches for studying the relationship between wine consumption, wine composition, and cancer have developed along two general lines: 1) The use of transgenic animal models of human disease and 2) the use of low-doses of chemical

carcinogens combined with Accelerator Mass Spectrometry (AMS) to evaluate the chemopreventive potential of wine components. In addition, we have used nutritionally adequate, well-defined amino acid based diets to control the nutritional status of animals in these studies. Finally, incorporation of test compounds or wine fractions into the animal diets at physiologic levels provides a nutritionally relevant way to evaluate their biological effects. The goal of this paper is to demonstrate the feasibility of these approaches for studying the cancer chemopreventive effects of wine and wine components.

MATERIALS AND METHODS

HTLV-1 Transgenic Mouse Model

Animals: The HTLV-1 transgenic mice were originally derived by microinjection of the long terminal repeat tax_1 gene construct into fertilized eggs from superovulated CD1 females crossed with C57BL/6-DBA2 F1 males (Nerenberg et al., 1987). Mice from this original founder line are maintained as a breeding colony at the University of California, Davis (Ebeler et al., 2002).

Mice were genotyped at ~7-10 d of age by tail biopsy and the *tax* sequence confirmed by Southern analysis (Southern, 1975; Feinberg and Vogelstein, 1984; Clifford et al., 1996) or by PCR analysis as described by Ebeler et al. (2002). After genotyping, mice containing the HTLV-1 tax_1 gene were randomly assigned to treatment groups and then housed in individual stainless steel wire bottom cages in a room with a 12-h light, 12-h dark cycle, a temperature of 20-23°C and a relative humidity of 50%. Mice were fed and

weighed daily and had free access to food and water throughout the study. The use and care of mice was approved by the our Institutional Animal Care Committee in accordance with the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Each day, the snouts, ears, feet, and tails of the mice were examined for the appearance of the first tumor. The age of the mouse on the day that a first tumor appeared was the age of tumor onset (tumor latency) for that mouse. Mice were killed within ~60 days of the time when a first tumor appeared to avoid complications of tissue wasting and cachexia.

Diets and Test Compounds: At weaning, all mice were fed a nutritionally adequate, amino acid based diet that was free of any test compounds before supplementation (Dyets Inc, #517802; Bethlehem, PA). The standard diet consists primarily of reagent grade chemicals and its composition can be precisely controlled (Ebeler et al., 1997). Test compounds were incorporated into the standard diet as described in Table 1. At approximately 20-27 d of age, the standard diet was replaced with the appropriate treatment diet while control animals remained on the standard diet.

Table 1. Dietary treatments for transgenic mice experiments.

	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**
Red Wine[1]	750 mL wine solids/kg diet; Total phenol content ~ 10 mmol GAE/kg diet[2]; Catechin conc = 900 μmole/kg diet[3]			750 mL wine solids/kg diet; Total phenol content ~ 11.46 mmol GAE/kg diet[2]; Catechin conc = 223 μmole/kg diet[3]

Catechin[4]		0.0, 0.5, 1.0, 2.0 mmol/kg diet	0.0, 1.0, 2.0, 4.0 8.0 mmol/kg diet	

[1]Wine for Experiment 1 was a Zinfandel, 1990 harvest, Sonoma Valley, California; Wine for Experiment 4 was a Sangiovese, 1994 harvest, Sonoma Valley. Wine solids were prepared by lyophilization of the wine to remove water and ethanol. Both wines were ~3 years old at onset of study (Clifford et al., 1996; Ebeler et al., 2002).
[2]Total polyphenol concentration was determined by Folin-Ciocalteau analysis (Zoecklein et al., 1995) and expressed as gallic acid equivalents, GAE.
[3]Individual monomeric polyphenols were identified and quantified by reverse-phase HPLC analysis (Ebeler et al., 2002).
[4](+)-Catechin was purchased from Fluka/Sigma (St. Louis, MO).

Data Analysis: Means (± SDs or SEMs) were calculated for all response variables. Differences in the age at first tumor onset between mice fed the different treatments were evaluated by Kaplan-Meier test (Experiment 1) or analysis of variance (Experiments 2, 3, 4) and Bonferroni-Dunn means-comparison tests (STATVIEW; Abacus Concepts, Berkely, CA). Dose-response relations were evaluated by using regression analysis (STATVIEW).

Chemical Carcinogen Model and Accelerator Mass Spectrometry

Animals: Male rats (35 days old; Fisher 344 strain; Harlan Sprague Dawley, Inc., Indianapolis, IN) were quarantined for 8 days then housed individually in wire mesh cages with *ad libitum* access to deionized water and a nutritionally adequate amino acid based diet (described above) for 4 days. The animals were then systematically divided into 5 groups of equal body weight and each group was randomly assigned to a control or a treatment diet. There were 5 rats in the control group and 2 in each of the treatment groups. The use and care of mice was approved by our Institutional Animal Care

Committee in accordance with the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Diets: Following the quarantine period, the rats were given *ad libitum* access, for 11 days, to diet that had been fortified with one of the following: **(a)** no fortificant (control); **(b)** 816 mg phenylethyl isothiocyanate (PEITC)/kg diet (PEITC ~99% purity, LKT Laboratories, St. Paul, MN); **(c)** 906 mg sulforaphane/kg diet (sulforaphane ~99% purity, LKT Laboratories, St. Paul MN); **(d)** 140 mg quercetin/kg diet (quercetin >99% purity, Fisher Scientific, Pittsburgh, PA), or **(e)** the lyophilized residue (dealcoholized and dehydrated wine solids) from 750 mL red wine/kg diet. The wine was a 100% Sangiovese harvested in October 1998, vinified on skins for 18 days. It was ~1.5 years old at the onset of the study. The doses of PEITC and sulforaphane were first dissolved in the corn oil that was then mixed with the rest of the amino-acid based diet components. The other test compounds were incorporated into the powdered diet and mixed well to yield a homogenous mixture.

Quercetin is a flavonoid found in wine and many other fruits and vegetables. It has been shown to increase the cellular concentration of reduced glutathione (Khandaju et al., 1999) and is a strong inhibitor of phenol sulfotransferases (Ghazali and Waring, 1999), enzymes that may be involved in PhIP bioactivation (Chou et al., 1995). PEITC and sulforaphane were studied for comparison purposes; these compounds are known to

influence mammalian Phase I and Phase II drug-metabolizing enzymes, and may be expected to alter carcinogen-DNA adduct formation (Talalay and Zhang, 1996).

Carcinogen Dosing: On the 10th day after beginning treatment diets (and following an overnight fast) each rat was dosed once with [^{14}C]PhIP by gavage. The [^{14}C]PhIP was added to corn oil to a concentration of 18 μg/mL and each rat received 5 μL/g body weight (90 μg [^{14}C]PhIP/kg body weight).

Tissue Collection and Analysis: One day (24 h) after dosing with the carcinogen, each rat was euthanized by CO_2 asphyxiation. Blood was collected by cardiac puncture into tubes containing heparin as anticoagulant and centrifuged at 3000 x g for 10 min to separate red cells. Liver, colon and prostate were excised promptly. Plasma, red cells, liver, colon and prostate were snap-frozen in liquid nitrogen and stored at –80°C until analysis.

Total concentrations of [^{14}C]PhIP in plasma, liver, colon, and prostate were analyzed by liquid scintillation counting as described by Dingley et al. (2003). The amount of PhIP/g tissue or plasma was calculated using the specific activity of the [^{14}C]PhIP (42 Ci/mol).

DNA was extracted from tissues as described by Dingley et al. (2003) using Qiagen DNA isolation columns (Qiagen Inc., Valencia, CA). DNA purity was determined from the ratio of the absorbency at 260 nm relative to the absorbency at 280 nm (A260 nm/A280

nm); all DNA in this study had a ratio of 1.7-1.8. Blood proteins, albumin and hemoglobin, were extracted as described by Dingley et al. (2003).

Because of the low-dose of carcinogen used in this study, [^{14}CPhIP] adducts to DNA and proteins were not detectable by liquid scintillation counting and required AMS for quantitation. For the AMS analysis, a portion of each DNA or albumin solution (262-1021 µg colon or liver DNA and 40-298 µg albumin from each animal or 5-41 µg pooled prostate DNA) was added to quartz tubes with 2 mg tributyrin added as a carrier to provide approximately 1 mg total carbon for AMS. Approximately 5-10 mg of hemoglobin from each animal was analyzed without the addition of carrier. Samples were prepared for AMS analysis by graphitization and the ^{14}C/^{13}C ratios measured by AMS, as previously described (Vogel et al., 1995; Roberts et al., 2000; Chiarappa-Zucca et al., 2002). The ^{14}C/^{13}C ratios were converted to adduct levels following the subtraction of the ^{14}C contribution from any added tributyrin and control samples.

Quinone reductase (QR) and glutathione S-transferase (GST) activity of hepatic tissue were analyzed as described by Dingley et al. (2003).

Data Analysis: Data were analyzed by ANOVA and differences among means were evaluated with Fisher's PLSD using STATVIEW software (Version 5.0.1, Abacus Concepts, Inc.). Statistical significance of differences between means of the groups was defined as a P value < 0.05. It was necessary to pool the prostate DNA from two rats in

each treatment in order to have sufficient tissues for analysis, therefore, only one value is shown for adduct formation in prostate.

RESULTS AND DISCUSSION

HTLV-1 Transgenic Animal Model

The HTLV-1 neurofibromatosis mouse model provides the opportunity to evaluate the molecular events involved in cancer initiation, promotion, and progression without the need for high doses of chemical carcinogens (Nerenberg et al., 1987; Hinrichs et al., 1987; Baird et al., 1992). HTLV-1 mice spontaneously develop external tumors (snout, ear, foot, tail) that can be easily viewed and assessed. The kinetics of tumor development in the HTLV-1 transgenic mice are well defined and consistent (Ebeler et al., 1997), and the tumors are similar to those occurring in human neurofibromatosis. Neurofibromatosis type-I (NF-1) is the most common dominantly inherited syndrome in humans that predisposes to neoplasia.

Using the HTLV-1 model we conducted a series of experiments to evaluate the effects of red wine and red wine components on tumor development. In the first experiment, we showed that red wine solids (alcohol free residue from red wine) significantly delayed tumor onset ($p < 0.05$) in the transgenic mice (Clifford et al., 1996; Ebeler et al., 1997). In this experiment, the median age of tumor onset was 91 d for mice fed the diet with wine solids compared with 66 d for sibling mice fed the same diet without the wine solids supplemented. The tumor-free period was extended by ~40% in mice fed the diet supplemented with wine solids.

In subsequent experiments (Experiments 2 and 3) we showed that dietary catechin, the major monomeric polyphenol in red wine, significantly delayed tumor onset in a positive, linear fashion when ingested at levels of 0.5 - 4mmol/kg diet ($r^2 = 0.761$, $p < 0.001$; Figure 1) (Ebeler et al., 2002). Furthermore, a wine with very low levels of catechin, but high total polyphenol concentrations, (see Table 1, Experiment 4) did not significantly influence tumor development; mean age of tumor onset was 81.5± 6.4 and 78.1 ± 4.0 d for the wine solids and control diets, respectively (Ebeler et al., 2002). These results suggest that specific dietary phenols, rather than total polyphenol concentrations may be important for cancer prevention and that the flavonoid catechin may have important biological effects in vivo.

Chemical Carcinogen Animal Model and Accelerator Mass Spectrometry

The transgenic animal studies have shown that tumor development due to a genetic pre-disposition can be significantly affected by environmental factors such as diet. However, a significant proportion of human cancers are also caused by exposures to low doses of chemical carcinogens. Therefore, the objective of this work was to present a protocol that uses physiological doses of carcinogens administered to test animals (i.e., rats) in order to monitor effects of chemopreventive agents on genotoxicity. Using a highly sensitive accelerator mass spectrometry technique (Vogel et al., 1995; Turteltaub and Dingley, 1998), we quantified the metabolic behavior and extent of DNA- and protein-adduct formation following exposure to low levels (mg/kg body weight) of the heterocyclic amine, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP).

The chemopreventive compounds tested in this study were consumed as an integral part of a nutritionally adequate amino acid-based diet. This diet was free of the test compounds (before supplementation) and its precise composition could be controlled and monitored reliably. All rats grew well and were healthy throughout the study.

In our preliminary studies we have observed that the wine polyphenol, quercetin, influenced the *in vivo* metabolic behavior and tissue distribution of [^{14}C]PhIP. Relative to a control diet, quercetin increased ^{14}C concentrations in the liver and prostate but decreased levels in the colon (Figure 2). For comparison, the two isothiocyanates, PEITC and sulforaphane, had no effect on tissue distribution of PhIP. The reasons for the differing tissue distribution of in diets containing quercetin compared to the other compounds are unknown and will require further study.

[^{14}C]PhIP-DNA adducts may be considered as biomarkers of cancer risk following PhIP exposure (Shields and Harris, 1991) and compounds which decrease adduct levels may act as potential cancer chemopreventive agents. Similarly, protein adducts in blood provide a measure of metabolic activation capability and may also be a useful surrogate markers for DNA damage in tissues (Skipper and Tannenbaum, 1990). Consumption of quercetin and wine solids did not alter [^{14}C]PhIP-DNA or [^{14}C]PhIP-protein adduct levels (Figures 3 and 4). In contrast, PEITC significantly decreased DNA- and albumin-adduct levels (Figures 3 and 4). Quercetin and wine solids did not induce the phase II detoxification enzymes, glutathione-S-transferase or quinone reductase (Table 2).

However, the PEITC-dependent reduction of PhIP-DNA adducts in liver correlated with

a 2 fold increase in the specific activity of these enzymes (Table 2).

Table 2. Enzyme specific activities in the liver of rats fed an amino acid-based diet supplemented with chemopreventives. Data are presented as means ± SE. Units for the GST and QR assays are μmol product formed per mg protein in the 100,000xg supernatant extract fraction. * Different from control at $P < 0.05$.

	Dietary Treatment				
	None (Control)	PEITC	Sulforaphane	Quercetin	Wine Solids
Liver GST	0.301 ± 0.007	0.596 ± 0.003* A 2.0-fold↑	0.274 ± 0.005	0.205 ± 0.036	0.219 ± 0.004
Liver QR	19.5 ± 1.0	52.1 ± 3.0* A 2.7-fold↑	31.0 ± 0.5* A 2.6-fold↑	21.6 ± 1.6	24.8 ± 0.4

Quercetin has been previously reported to strongly inhibit PhIP N-hydroxylation in rat

and human liver microsomes (Hammons et al., 1999). It has also been shown to have

significant QR- and GST-inducing activity in cell culture (De Long et al., 1986; Kurata et

al., 1992; Williamson et al., 1996). Yet in the present *in vivo* feasibility study, quercetin

did not affect adduct levels or liver enzyme induction. Although liver and prostate tissue

levels of PhIP were higher in rats consuming diets containing added quercetin, the levels

may not have been high enough to induce the enzymes GST or QR. These results point

to important differences between *in vivo* and *in vitro* studies, and indicate that absorption

and metabolism of test compounds are important variables that must be considered when

evaluating chemopreventive activity. The quercetin concentration used in this study was

~3 - 10 times higher than average quercetin levels in red wine (Ritchie and Waterhouse,

1999; Ebeler et al., 2000; Clifford et al., 1996) and may explain why red wine solids

alone had no effect on tissue distribution, adduct levels, or enzyme induction in this study. However, levels of quercetin and structurally related flavonoids in some wines and in other fruits and vegetables (e.g., onions) may reach the levels tested in this study (Singleton, 1976; Hertog and Hollman, 1996; Ritchie and Waterhouse, 1999). Typical human intake of quercetin is estimated to range from 6 – 30 mg/day (Hertog and Hollman, 1996).

Our studies are based on a limited number of animals and further work is needed to fully evaluate the effects of these dietary constituents. However, we have shown the feasibility of using low-dose carcinogens combined with accelerator mass spectrometry to quantify metabolic behavior and extent of adduct formation for compounds with chemopreventive potential.

SUMMARY

We have developed highly sensitive protocols using chemically defined diets that can be controlled and supplemented with natural foods, food fractions, or pure compounds to evaluate the effects of dietary interventions on both genetic and chemically-induced cancers. Using these techniques we have shown that red wine (alcohol-free residue) and wine components can have significant chemopreventive potential at physiologic levels *in vivo*.

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List of Figures

Experiment 2

Experiment 3

Age when first tumor appeared, days

Dietary catechin, mmol/kg

Treatment

Legend:
- Liver (□)
- Colon (▨)
- Prostate (■)

Treatments: Control (none), PEITC, Sulforaphane, Quercetin, Wine solids

Y-axis: DNA adduct level (adducts/10^{12} nucleotides) — 0, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000

Treatment

ng PhIP/g protein

Legend:
- Albumin (filled)
- Hemoglobin (open)

X-axis (Treatment): None (control), PEITC, Sulforaphane, Quercetin, Wine solids

Y-axis: 0, 2, 4, 6, 8, 10, 12, 14, 16

Experiment 2

Y = 76.37 + 42.06 X; R^2 = 0.513
P = 0.001

Experiment 3

Y = 73.2 + 8.36X; R^2 = 0.761
P < 0.0001

Dietary catechin, mmol/kg

Age when first tumor appeared, days